GENE REGULATION IN TELEOSTS BY ESTRADIOL AND ESTROGEN MIMICS

By

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Estradiol plays a critical role in homeostasis, growth, and reproduction. These physiological effects of estradiol are very tightly regulated at the level of gene transcription. This regulation is vulnerable to perturbation by environmental contaminants that mimic estradiol. The biological response over time and with dose by estrogenic chemicals helps determine the relative risk of hormone-sensitive disease and cancer in all organisms including humans. This dissertation investigates the effects of estradiol and environmental estrogen mimics on gene expression in sentinel species.

Estrogen receptor, a nuclear transcription factor, once bound by specific chemicals binds to specific DNA regulatory sequences to control gene transcription. Two teleost species, largemouth bass and sheepshead minnows, with unique characteristics were chosen to evaluate the activation of the estrogen receptor-mediated pathway. In fish, both vitellogenin and estrogen receptors are known to be induced by estradiol. In largemouth bass the hypothesis tested was the coordinated induction of estrogen receptor and vitellogenin mRNA over time after acute exposure to estradiol. An

in vitro method was also developed using liver cells isolated from largemouth bass. Future studies using this model can be used to evaluate the mechanisms behind the differential induction of mRNAs.

Sheephead minnow were used to establish and test an *in vivo* bioassay for estrogenic chemicals. The dose and time-dependent induction of vitellogenin mRNA and protein were characterized after exposure to estradiol, ethinylestradiol, diethylstilbestrol, nonylphenol, methoxychlor, or endosulfan. The decrease of Vtg mRNA and protein levels induced by estradiol or nonylphenol following transfer to clean water was also determined.

This research established the baseline experimental responses of estrogen receptor and vitellogenin mRNAs to estradiol and various estrogen mimics using different routes of exposure. By differential display additional genes under estrogen control were identified in these two teleost species, including the zona radiata proteins, transferrin, and a protein disulfide isomerase, ERp72.

CHAPTER 1
INTRODUCTION AND LITERATURE REVIEW

Background

The Endocrine Disruption Hypothesis

Beginning in 1962 with Rachel Carson's landmark publication, Silent Spring, the general public became acutely aware that industrial progress did not operate in a bubble, but held a silent environmental consequence that was growing unchecked (Carson, 1962). In the years to follow, the United States created the Environmental Protection Agency and placed new emphasis on pesticide regulation and food safety. Then in 1991, the term "endocrine disruptor" was coined at the first of a series of scientific work sessions known as the Wingspread Conferences in Racine, Wisconsin. Simply put, the endocrine disruption hypothesis is that some chemicals in the environment mimic estrogens and other hormones, and these can interact with endogenous endocrine systems with unnatural repercussions.

Supporting Environmental Evidence

Epidemiological studies of humans and observational research of wildlife following environmental exposure have provided the most realistic evidence for endocrine disruption. In humans, *p,p'*-DDE (*p,p'*-dichlorodiphenyldichloroethylene) concentrations in breast milk have been linked to significantly shortened duration of lactation (Rogan et al. 1987). Studies of polychlorinated biphenyl (PCB)-contaminated rice oil in Yusho, Japan and Yu-Cheng, Taiwan demonstrated a unique "fetal PCB syndrome" in infants of exposed pregnant women (Hsu et al. 1985, Rogan et al. 1988,

Yamashita & Hayashi 1985). This syndrome included low birth weight, increased mortality, rocker bottom heel, and abnormal calcification of the skull among other signs. In the North American Great Lakes region deficits in neurologic development of children were positively associated with prenatal exposure of maternally consumed PCB-contaminated fish. Potentially the most significant impact on human health is on male reproduction. The issue is two-fold, male reproductive disorders appear to be increasing across the world and over a large period of time beginning with the introduction of these environmental contaminants. If this is true, are these environmental contaminants responsible? Currently under investigation, this issue is an area of tremendous controversy. Several specific disorders are under scrutiny: cryptorchidism, hypospadias, testicular cancer, and several parameters concerning poor sperm health (Carlsen et al. 1995, Toppari et al. 1996). None have been conclusively or directly linked to endocrine disrupting contaminants, but research continues in this area.

Evidence for suspected endocrine disruption is perhaps most convincing in wildlife. Fish are useful indicators of ecosystem contamination since they are exposed to all the pollutants in a particular drainage basin due to runoff and erosion. Municipal and industrial (textile, pulp mill, etc.) effluents have been associated with endocrine modification and reproductive disruption in fish. These effluents have been reported to be responsible for testes containing oocytes (intersex) (Harries et al. 1997, Jobling et al. 1998) and increased female-specific proteins in male fish (Folmar et al. 1996, Purdom et al. 1994). Reduced plasma sex steroids, gonadotropins, egg and gonad size have been reported in effluents from bleached kraft mills (Van Der Kraak et al. 1992). The Great Lakes region in North America serves as another example of disruption in wildlife. The high levels of PCBs, dioxins, and DDT in fish tissue have served as a model of

bioaccumulation and persistence of these contaminants. They have been held collectively responsible for decreased population in various fish species (lake trout and salmon) (Guiney et al. 1996, Zabel et al. 1995). These chemicals in the fish have gone up the food chain to affect bird populations as well. Cormorants, gulls, and terns have been shown to have the most significant population decline (Allan et al. 1991, Fox et al. 1991, Giesy et al. 1994), as well as skewed sex ratios in gulls (Hunt et al. 1980). Decreased larval survival and maturation of English sole in the Puget Sound is another example (Casillas et al. 1991, Johnson et al. 1988). In Florida, investigators have shown decreasing population and reproductive abnormalities in alligators from Lake Apopka (Guillette et al. 1994, Rice et al. 1996, Woodward & Moore 1990).

Due to the scientific, political, and social questions raised by these observations and ideas, the United States amended the Safe Drinking Water Act (PL 104-182) and the Food Quality Protection Act (PL 104-170) in 1996 to require testing of potential xenoestrogens. In addition, because of the complex and unknown nature of how these chemicals affect biosystems, research into possible mechanisms of action is a priority (Kavlock et al. 1996). Both mammalian and non-mammalian vertebrates have been used extensively for *in vitro* and *in vivo* laboratory research. Because of the complexity of the endocrine system there is no single test to determine if a compound will cause adverse hormonal activity. To complicate matters much of the basic comparative endocrinology and physiology among relevant species is not well understood, especially in response to xenobiotic insult.

Supporting Laboratory Evidence

Many laboratory experiments were done to investigate potential mechanisms of hormonal disruption. Experiments duplicating the known adverse effects of

diethylstilbestrol (DES) in humans showed how sensitive reproductive and developmental end points are to xenobiotics acting through estrogen mechanisms (Herbst & Bern 1981, McLachlan et al. 1975, Newbold, 1995). This has opened up the possibility of other estrogen-mimics having similar or otherwise adverse effects on humans and wildlife. The effects measured in adults are often only transient, whereas developing organisms can be permanently imprinted and may be more sensitive to dose; therefore choosing the appropriate developmental window of exposure is important. It is very difficult to connect cause and effect even in laboratory experiments since the end point effects are usually significantly removed in time from the exposure. The identification of appropriate end points or markers is equally difficult for this reason.

In mammalian models there is good evidence for disruption following reproductive or developmental exposure to estrogenic chemicals. Reduced seminal vesicle and prostate weight in rats exposed to *o,p'*-DDT (*o,p'*-dichlorodiphenyltrichloroethane) (Gellert et al. 1974), blocked implantation of embryos by methoxychlor (Cummings, 1990), and changes in territorial behavior of mice exposed prenatally to *o,p'*-DDT, DES, or methoxychlor (vom Saal et al. 1995) are just a few examples. Sharpe et al. reported a reduction in daily sperm production in rats following butyl benzyl phthalate and octylphenol (Sharpe et al. 1995). Rats had increased prostate weights after gestational exposure to very low levels of bisphenol A (Nagel et al. 1997) which is very similar to the effects exhibited by DES (vom Saal et al. 1997). When attempting to identify doses in the range of human exposure (Brotons et al. 1995, Olea et al. 1996), these particular studies are in that range.

In wildlife, studies relevant to environmental exposures have been conducted to identify potential effects of polluted ecosystems. Some examples are significant

decreases in the rate of testicular growth in rainbow trout following alkylphenol exposure (Jobling et al. 1996), altered sexual differentiation following alkylphenol exposure resulting in feminization of male carp (Gimeno et al. 1998b), and altered plasma steroid hormone levels and poorly organized testes in Lake Apopka alligators (Guillette et al. 1994). Xenobiotic exposure can induce skewed sex ratios in turtles (Bergeron et al. 1994, Crews et al. 1991) and eggshell thinning in birds (Cooke, 1973) as well. Despite reported examples of endocrine disruption, the mechanisms are still unclear. In the case of wildlife, there is not enough baseline or basic research to support the necessary toxicological studies.

Xenobiotics with Estrogenic Activity

One of the most puzzling aspects of xenoestrogens is their common ability to bind the same receptor despite having very uncommon molecular structures. Some are not even steroids, such as diethylstilbestrol. Structure-activity relationships to build predictive models have not been very successful for this reason. The promiscuity of the estrogen receptor (ER) is well known, but not well understood. It isn't even clear if all these xenoestrogens even bind the receptor in the same binding site. In general, most of these xenoestrogens are roughly one thousand times less potent than the endogenous ligand, E_2, using binding affinity for the ER. The only exceptions are the pharmaceutical estrogens. New classes of compounds that elicit estrogenic activity are still being discovered. See Figure 1-1 for representative structures of different families of suspected xenoestrogens.

Pharmaceutical Estrogens

Diethylstilbestrol (DES) and ethinylestradiol (EE_2) are two prominent synthetic chemicals that were custom tailored to function similarly to the endogenous ligand, 17β-estradiol (E_2). Ethinylestradiol is currently used in oral contraceptives, and is commonly prescribed at a dose of 20 to 50 micrograms per day. It also has a slower rate of metabolism than the endogenous ligand, and much of it is eliminated from the body. So not too surprisingly, a significant amount of the parent compound (EE_2) is released into the environment in municipal waste effluent (Aherne & Briggs 1989, Desbrow et al. 1998, Tabak et al. 1981). DES, once taken during gestation to prevent miscarriage, is now known to produce adverse effects and is no longer used (Dieckmann et al. 1953). The mechanism of action of DES in humans and wildlife is well studied, and continues to be used as a model compound for xenoestrogen actions (Herbst & Bern 1981, McLachlan et al. 1975). Both diethylstilbestrol and ethinylestradiol are approximately equipotent to E_2, and have similar mechanisms despite divergent structures.

Organochlorine Compounds

This is largely a class of persistent PCBs and pesticides that are ubiquitous in the environment. Even though the United States has banned the use of PCBs and DDT since 1977 and 1973 respectively, these compounds are still present in significant amounts in this country. Concentrations of PCBs, DDT, chlordane and hexachlorocyclohexanes have been detected worldwide (Iwata et al. 1993) and in rainwater (Rapaport et al. 1985) which helps explain the enormous distribution. Many of these chemicals are considered estrogenic by *in vitro* assays such as the E-Screen (Soto et al. 1994, Soto et al. 1995), or by *in vivo* bioassays (Ecobichon & MacKenzie 1974). Some of these organochlorines such as methoxychlor and *o,p'*-DDT are considered estrogenic by most screening assays

and have shown mechanisms of action and whole-body effects similar to E_2 (Bitman et al. 1968, Cummings, 1997).

Phenolics

Alkylphenol ethoxylates, 4-alkylphenols, bisphenol A, and some other types of PCBs fall into this category of proposed xenoestrogens. Alkylphenols are used as surfactants and emulsifiers in numerous industrial and commercial applications; 360,000 tons were produced worldwide in 1988 (Nimrod & Benson 1996). They are also components of herbicides, cosmetics, paints, and as spermicides in topical contraceptives. (Nimrod & Benson 1996) These compounds are thought to be partially responsible for observed estrogenic effects of municipal and industrial plant effluent, as they have been measured up to hundreds of microgram per liter (Ahel & Giger 1985, Naylor et al. 1992). They have been shown by most *in vitro* and *in vivo* assays to induce effects similar to E_2 (Jobling & Sumpter 1993, Jobling et al. 1996, White et al. 1994). Bisphenol A is a chemical intermediate for various industrial products including polymers, resins, dyes, and flame retardants; it is also used in dental sealants and various plastics. Its estrogenicity is convincing in both *in vitro* and *in vivo* assay for both rodents and wildlife (Brotons et al. 1995, Krishnan et al. 1995, Nagel et al. 1998, Soto et al. 1995, Soto et al. 1997, Sumpter & Jobling 1995). *In vivo* bioassays have shown the ability of some phenolic PCBs to exhibit estrogenicity as well, particularly 2',4',6-trichloro-4-biphenylol and 2',3',4',5'-tetrachloro-4-biphenylol (Jansen et al. 1993, Soto et al. 1995, Young et al. 1995).

Naturally Occurring Compounds

Xenobiotics with potential estrogenic activity do not have to be synthetic as mentioned above. Naturally occurring compounds in plants as well as fungal metabolites are foreign to our body, yet some appear to mimic the activity of E_2 (e.g. phytoestrogens). Several of these chemicals are capable of binding to the ER and testing positive in recombinant-reporter assays (Miksicek 1995, Verdeal et al. 1980). For example, genistein (flavonoid), coumestrol (flavonoid-derived compound), and zearalenone (fungal metabolite) all exhibit estrogenic activity as measured by two or more assays. Wood-derived phytoestrogens, such as beta-sitosterol, could partially account for the proposed estrogenic activity in pulp and paper mill effluents (Mellanen et al. 1996, MacLatchy & Van Der Kraak 1995). Even though we know relatively little about these compounds, there is frequent dietary exposure to humans and infants (soy milk). Therefore, more research is needed to better understand what dosages have pertinent biological activity in exposed biota.

Other Compounds

It seems that new groups of chemicals are constantly being recognized as potential endocrine disrupting chemicals. Most recently the phthalates have been added to the list of suspected xenoestrogens. These are chemicals used as plasticizers for polyvinyl chloride and coatings, as cosmetic components, as solvents, or as a leak detector. Butylbenzylphthalate, butylated hydroxyanisole, and di-*n*-butylphthalate are the most interesting. In fact, they seem to bind the ER and positively respond to E-Screen and gene induction assays, but without *in vivo* estrogenic responses (Jobling et al. 1995, Soto et al. 1995). Rather, some evidence suggests they may actually be acting as anti-androgens in male reproductive assays (Mylchreest et al. 1998). However unlike the

antiandrogen flutamide, phthalates do not seem to block the androgen receptor to elicit antiandrogenic action (Mylchreest et al. 1999).

Fish Models

The basic features of endocrine-mediated pathways are known to be evolutionarily conserved in most vertebrates. Despite some unique genes and specific sensitivities to regulation, the general hormone signaling pathways are thought to be similar between fish and mammals. When trying to establish cause and effects of environmental endocrine disruption, fish have been proven to be one of the more affected species. This diagnosis stems from problems in reproduction and development of sensitive species in isolated regions. A couple of well known examples include population declines of lake trout, bloaters, salmon, lake herring, striped bass, and walleye in the North American Great Lakes region (Guiney et al. 1996, Zabel et al. 1995), as well as decreased larval survival and maturation of English sole in the Puget Sound site (Casillas et al. 1991, Johnson et al. 1988). In some Florida lakes, there is evidence for alligator reproductive impairment (Guillette et al. 1994, Rice et al. 1996, Woodward & Moore 1990) and some preliminary data in our labs suggests declines in some fish species, especially largemouth bass, from those same lakes. In general, fish can serve as relevant biologically-impacted models of environmental health. This is because of their large numbers, limited range, sensitivity, and their proximity to many potential environmental exposures. Being aquatic animals, there are various routes of exposure (gills, food, & dermal) present in our lakes and rivers. These are the same lakes and rivers that we drink from and dump our wastes into.

Fish can serve as sentinel species illustrating the exposure to and effects of current levels of pollution. In addition to sentinel species in the wild, fish have also been used extensively in laboratory studies. The medaka, fathead minnow, goldfish, killifish, sheepshead minnow, and zebrafish for example are small, easily maintained fish ideal for large-scale exposure studies. The zebrafish for example serves as a model where the entire genome has been sequenced. Another larger fish model is the rainbow trout, where a plethora of background data is available.

For the studies presented in this research a large, environmentally impacted freshwater fish model (largemouth bass), and a small laboratory estuarine model (sheepshead minnow) were chosen. One of the major differences is size; larger fish provide more tissue and material to investigate per sample, allowing for unique experimental designs such as primary organ culture. Large numbers of small fish on the other hand are easy to maintain in the laboratory, making realistic exposures and sample sizes easier to work with. The comparative biology of these two models is also useful when studying aspects of endocrinology and reproduction. Largemouth bass reproduction is synchronized and spawns annually, this biological feature enables us to chart baseline physiology and detect any disruption by xenobiotics. Sheepshead minnow, on the other hand, is a fractional spawner that serves another purpose in monitoring full life cycle and generational responses in the laboratory, but within a much shorter period of time. I would expect that these biological differences result in species-specific sensitivities to various xenobiotics.

Studies using these model organisms and sentinel species as bioindicators of the state of the environment are important to detect various biologically-active pollutants and their potential mechanisms of action. The presence of specific xenobiotics, if not abated,

can continue to accumulate and have the potential to disrupt higher-order organisms such as humans. The scenario is not new, Rachel Carson predicted such a state over 35 years ago in her book Silent Spring (Carson, 1962), and this was echoed recently in the context of endocrine disruption by Theo Colborn in the book Our Stolen Future (Colborn et al. 1997).

Vitellogenin

One of the reasons fish provide such valuable information on environmental estrogens is the endogenous production of the protein vitellogenin (Vtg). Vtg is a female-specific yolk precursor protein in oviparous (egg laying) vertebrates (Mommsen & Walsh 1988). Vtg is a large phospholipoglycoprotein that is thought to be conserved in function across oviparous vertebrates, but the amino acid sequence is not very well conserved across species even from the same taxa. Vtg from different species share small patches of conserved sequences amid larger segments that vary considerably. Vitellogenesis is initiated through environmental signals and the hypothalamic-pituitary-gonadal axis. These signals from the brain (gonadotropins) tell the gonads to synthesize 17β-estradiol from testosterone (aromatization). Estradiol is secreted into the blood and diffuses into the liver. Once in the liver it is bound by estrogen receptors which interact with chromatin to expose DNA-binding sites. This ultimately induces transcription and translation of the Vtg gene. Once translated, Vtg undergoes extensive post-translational modification and is secreted into the blood. It is transported as a dimer and is selectively taken up by the developing oocyte and specifically cleaved into the egg yolk proteins, phosvitin and lipovitellin (Figure 1-2) (Mommsen & Walsh 1988).

Model of Hormonal Regulation

Vitellogenin historically has been used as a model for steroid-induction of gene activation (Tata & Smith 1979). It is keenly specific for estrogens since the 5'-promoter region of the gene has consensus estrogen response element sequences for ligand-bound estrogen receptor protein complexes (Burch et al. 1988). Males do not express Vtg at any appreciable level, but it can be induced artificially, and is thus a useful tool for the study of cell and molecular biology of gene expression as regulated by E_2 (Wahli et al. 1981). For basic science, it has been used as a tool for characterizing hormone induction profiles (Bowman et al. 2000, Ryffel, 1978). In fact Vtg mRNA has also been shown to exhibit differential half-lives in the presence of estrogen, and therefore provides an endogenous mechanism for characterizing inducible mRNA stability (Brock & Shapiro 1983). There is evidence to suggest this post-transcriptional mechanism of inducible mRNA stability is susceptible to disruption by xenoestrogens (Ratnasabapathy et al. 1997).

Biomarker of Estrogenicity

Vitellogenin has been used as a model to answer many questions both in basic science and for the current topic of environmental endocrine disruption. Vtg is perfectly suited as an *in vivo* biomarker of estrogen exposure (Denslow et al. 1999b, Heppell et al. 1995, Palmer & Selcer 1996). The presence of Vtg in male or juvenile oviparous vertebrates indicates prior estrogen exposure. This is particularly useful in wild populations of fish present in polluted waters. By quantifying the amount of Vtg in male fish *in vivo*, people have identified the presence of an estrogenic component in industrial and municipal effluents (Folmar et al. 1996, Harries et al. 1999, Purdom et al. 1994). Currently, it is not clear if there are adverse effects of small amounts of Vtg in males. There is some evidence that environmentally-relevant levels of Vtg in male flounder can

result in hepatocyte hypertrophy, disruption of spermatogenesis and kidney damage (Folmar et al. 2001). In addition to plasma Vtg protein, Vtg mRNA can be used to identify and characterize acute or chronic effects at the molecular level (Bowman et al. 2000, Hemmer et al. 2001). Quantification of Vtg mRNA can provide more sensitive insight into timing and mechanisms of hormonal disruption.

Estrogen Receptor Mediated Pathway

Steroid hormone-mediated actions are critical for reproduction, the stress response, growth & development of individuals, homeostasis, and much more. All of the above events are vulnerable to disruption and/or modulation by xenobiotics. However, our understanding of the mechanism of such actions is confounded by crosstalk among different steroids, their respective receptors, organ specificity, cellular environment, and nonsteroidal signal transduction. Historically, the estrogenic response is the best-understood steroidogenic pathway of gene activation. This response is thought to be mostly directed through the ER. There remain many unresolved issues regarding gene regulation and the dependence on tissue-specific intracellular constituents that dictate the differential expression of estrogen-responsive genes (Zacharewski, 1998). In characterizing the estrogenic response one must go from physiological effects seen *in vivo* to isolated molecular events that have the capacity to dictate such responses.

The simple ER-mediated pathway starts with the translocation of the lipophilic ligand (E_2) into the cell by simple diffusion (Figure 1-3). Once in the nucleus it binds to the ER (King & Greene 1984), displacing its chaperone heat shock proteins. On binding of the ligand, the receptor undergoes a conformational change that allows the formation of homodimers (Fritsch et al. 1992, Kumar & Chambon 1988) (and possibly

heterodimers) (Glass, 1994). This ligand-bound ER dimer complex then binds to a specific inverted repeat sequence on the DNA known as the estrogen response element (ERE) (Kumar & Chambon 1988, Mader et al. 1993). This *cis* element has been found near almost all estrogen-regulated genes (Glass, 1994, Lucas & Granner 1992). This bound complex is thought to recruit the necessary co-factors to initiate transcription of estrogen-inducible genes (Truss & Beato 1993). Once all the appropriate signals are present, synthesis of target gene mRNAs begin, followed by the translation of that coding sequence to active proteins that contribute to the estrogenic response. Known target genes include: pS2 and cathepsin D in human cells lines (Pilat et al. 1993), *c*-fos, *c*-myc, and *c*-jun in rats (Weisz & Bresciani 1993), apolipoprotein E in mouse (Srivastava et al. 1997), and vitelline envelope proteins (Larsson et al. 1994), ER, and VTG in fish (Anderson et al. 1996).

Although the model for this seemingly simple pathway is mostly correct, there are still a plethora of regulatory factors at each step that are only beginning to be understood. First is the control of free estrogen or xenoestrogen in the plasma, either through biosynthesis or differential binding by sex hormone binding globulin (SHBG), a plasma protein (Joseph, 1994). Then there is the ER itself that has been well characterized, but it remains unclear how the protein itself is regulated. There is good evidence that the conformational change of the ER that allows DNA binding is partially ligand-specific (Fritsch et al. 1992, Paech et al. 1997). Recently, the presence of an ERβ isoform was discovered in rats (Kuiper et al. 1996), and now human, fish and mouse homologues have been found as well. There is now data supporting how ERα and β work differently (Paech et al. 1997), and in some cases together (Pace et al. 1997), with multiple ER splice variants complicating matters (Peterson et al. 1998). Interactions with various orphan

steroid receptors are also beginning to complicate the picture of estrogen regulation (Giguere et al. 1988). Very recently a third receptor, ERγ, identified in ovaries of Atlantic croaker was discovered (Hawkins et al. 2000). With unique tissue specificity and phylogenetic diversity, the presence of a third ER promises to raise more questions than answers.

One of the more contemporary models to explain the diversity of the estrogenic response is the idea that various *cis* elements of a particular target gene bind individual transcription factors and/or steroid receptor complexes. This transactivational complex is responsible for a particular organ and gene-specific response (Tsai & O'Malley 1994). There have been several of these proteins identified that act as either co-activators (SRC-1, GRIP1, AIB1) or as co-repressors of the ER-mediated response (Shibata et al. 1997). Alternative pathways of ER activation involving membrane receptors have also been suggested (Loomis & Thomas 2000, Watson et al. 1995). One of the crosstalk scenarios is epidermal growth factor and its tyrosine kinase receptor activating the signal transduction cascade to influence the ER-mediated pathway (Ignar-Trowbridge et al. 1992, Kato et al. 1995). Many of these potential pathways involve very specific phosphorylation of the receptor and how this regulates gene activation (Arnold et al. 1995, Le Goff et al. 1994). Recent evidence suggests the involvement of other pathways, such as calcium release and mitogen-activated protein kinase activation (Improta et al. 1999, Morley et al. 1992). These types of regulatory features are not well defined yet but are an area of active interest in steroid research.

Identifying possible mechanisms of how xenoestrogens can alter physiological events is a complex task. In addition to the gross observations made in the field and the laboratory following exposure, it is critical to understand the molecular events

responsible behind the scenes. It will be at this level that the interaction of environment and biology will begin to prove or disprove the endocrine disruption hypothesis. The following chapters will provide a molecular approach to how two relevant aquatic organisms respond to estrogen exposure *in vivo*. The establishment of an *in vitro* primary hepatocyte culture system from largemouth bass enables the direct testing of estrogen stimulation of ER and Vtg mRNA synthesis. This will provide the necessary information on the basic endocrinology to support future experiments testing the endocrine disruption hypothesis.

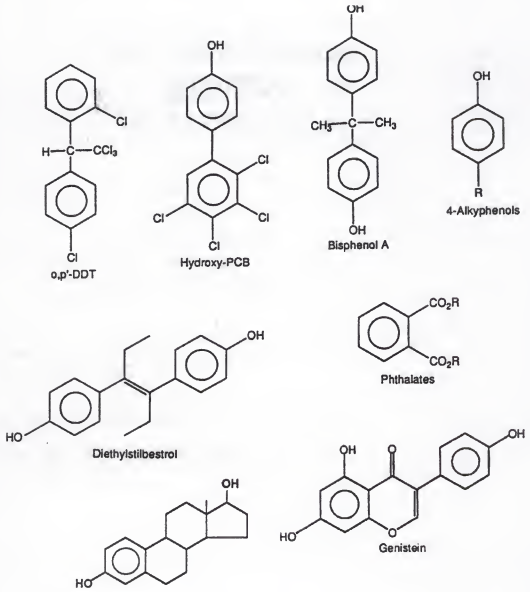

Figure 1-1. Representative chemical structures of different families of suspected xenoestrogens.

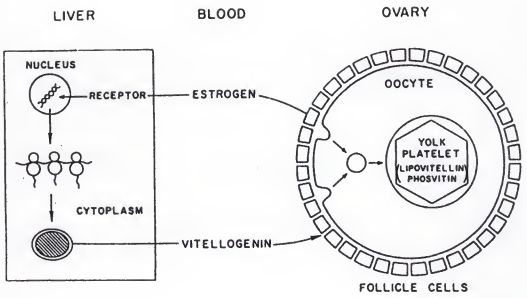

Figure 1-2. Relationship between the liver and ovary during vitellogenesis in females. This shows how estrogen induces synthesis of hepatic vitellogenin which is secreted into the blood for delivery to the developing oocyte (Adapted from Tata and Smith, 1979).

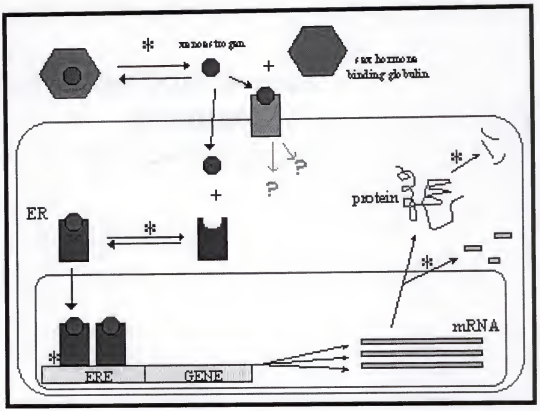

Figure 1-3. Simplified scheme of the estrogen receptor mediated pathway. Estrogen passively diffuses into the hepatocyte and is bound by the ER in the nucleus or cytoplasm. Bound and dimerized receptors bind to specific DNA sequences to initiate transcription of specific genes such as vitellogenin. Asterisks indicate possible positions of xenoestrogen disruption, including other protein-protein interactions in the nucleus.

CHAPTER 2
COORDINATE *IN VIVO* GENE EXPRESSION IN LARGEMOUTH BASS (*MICROPTERUS SALMOIDES*) AFTER ACUTE ESTRADIOL EXPOSURE

Introduction

If the ultimate goal is to understand and protect ecosystem health, *in vivo* experiments are practically impossible to replace with *in vitro* assays. Even though there are numerous limitations to *in vivo* experiments, such as smaller sample size, cost, and lack of sensitivity, their benefits often outweigh the costs. For example, a single experiment can provide important information on absorption, distribution, metabolism, elimination, acute vs. chronic dose, organ weight and morphology, gene and protein expression, interaction and effects on or with endogenous hormones, mortality, and reproductive success. Not to mention all the unknown cellular proteins and pathways that may actually be responsible for the evaluated end points. The basic scientific understanding of endocrine-active compounds and how they may mimic hormone-specific responses requires whole-animal investigation.

To investigate estrogen-mediated mechanisms of endocrine disruption, there are several long-standing hormone-specific assays. In mammalian systems these include vaginal cornification (Allen & Doisy 1923, Edgren, 1994), vaginal epithelial cell proliferation (Martin & Claringbold 1958), vaginal opening (Allen & Doisy 1924, Edgren et al. 1966), vaginotrophic response (Folman & Pope 1966), uterotrophic response (Rubin et al. 1951), uterine glycogen deposition (Bitman & Cecil 1970), and uterine estrogen-withdrawal bleeding (Schane et al. 1972). For environmentally-impacted animals such as fish there are few estrogen-relevant end points. One is vitellogenin (Vtg) synthesis in

male oviparous species (Folmar et al. 1996). Other assays include altered sex steroids (Van Der Kraak et al. 1992), organ weights (Munkittrick et al. 1994), and the presence of ovo-testis by histopathology (Gimeno et al. 1998a, Jobling et al. 1998).

Coordinate Gene Regulation

The integration of signals that ultimately control the estrogenic response is complex, therefore it is important to characterize the response at the transcriptional and translational levels to better understand the physiological outcome, while still accounting for specific gene regulation. Most estrogenic responses are elicited via *de novo* synthesis of specific estrogen-regulated genes (Tsai & O'Malley 1994, Carson-Jurica et al. 1990), including the estrogen receptor (ER) itself in oviparous vertebrates. Characterizing the transactivation of specific genes following exposure is important to understand the coordinated tissue and species-specific protein-protein and protein-DNA interactions. This is partially why gene expression monitored *in vivo* is so important.

On closer examination of transcriptional events, multiple waves of responses are seen following a single hormone exposure. Specific tiers of transcriptional activation exist after a single exposure to a hormone: primary, secondary, and delayed primary responses (Dean & Sanders 1996). A primary response is defined as those mRNAs that are up regulated directly by exposure to a hormone. A secondary response is defined as those mRNAs that are not induced directly by the hormone, but rather by protein products of the mRNAs originally induced. A delayed primary response refers to mRNA transcription that results both from a direct interaction of the hormone and additional factors that are products of the primary response (Figure 2-1). The delayed primary response would be delayed by several hours relative to the primary response. Induction of vitellogenin mRNA might be considered a delayed primary response. These types of

varied transcriptional responses have been observed in several different systems and species (e.g., chicken ovalbumin (Dean & Sanders 1996) and rat $\alpha_{2\mu}$-globulin (Chan et al. 1991)). The ordered sequence of transcriptional events is expected to be important for homeostasis and certainly during reproduction and development. Disruption of these events is thought to be very sensitive to hormonally active agents.

The estrogenic response in oviparous (egg-laying) animals may represent one model to study this transcriptional network. The up regulation of ER mRNA and protein is a primary response, as it is immediate and sensitive following estradiol (E_2)-exposure. The vitellogenin gene found in these animals is thought to elicit both a small primary response and a larger delayed primary response (Pakdel et al. 1990). It is believed that the additional ER synthesized in the primary response is responsible for the delayed response seen with Vtg (Pakdel et al. 1991). A secondary response is seen with genes that do not directly bind intracellular receptors, but instead are activated or induced by one of many potential intermediary proteins (Dean & Sanders 1996). These intermediary proteins could consist of transcription factors, kinases, mRNA stability proteins, or intracellular cofactors. The classic example of this tiered response was first shown by a steroid (20-hydroxyecdysone)-induced transcription factor that induced waves of polytene chromosome puffing in Drosophilia (Thummel, 1995). At this time, the evidence for estrogenic secondary response genes that are a direct effect of these intermediary proteins is scarce, but remains an area of active research. Part of the problem with studying estrogen target genes across species is that very few have been discovered. Most of what is understood concerning E_2 is its proliferative effects in reproductive tissues and its signal to synthesize vitellogenin (precursor to egg yolk

protein). Because of this, Vtg in frogs, chickens, fish, and birds have been used as model systems to study hormone induction (Ryffel, 1978).

By studying the regulation of ER and Vtg gene induction following E_2-exposure it is possible to learn more about the establishment of complex gene networks in a system. In addition to understanding how known genes are coordinately regulated, the discovery of novel or previously unrecognized estrogen-regulated genes may lend insight into the dynamics of gene networks. One mechanism for identifying such unknown estrogen-regulated molecules is differential display (Liang & Pardee 1992). Samples collected over time following an acute exposure, once analyzed by differential display, could lead to such discoveries.

Largemouth Bass

Largemouth bass (*Micropterus salmoides*) (Figure 2-2) is a native freshwater fish found in the Great Lakes region and the Mississippi River Basin from southern Quebec down to the Gulf of Mexico. In addition to its large natural distribution, it is stocked in over 30 states as a game fish. Because it is a piscivorous predator and can grow quite large, it is usually at the top of the fish food web in its natural territories. This capacity to embody much of the freshwater fish population and its suspected sensitivity makes it a model sentinel species. In recent years fish biologists and researchers have documented declining numbers in local Florida lakes. In addition, stocked fish reproduction is now suffering in reclaimed wetlands due to unknown circumstances in the environment. One hypothesis is the residual presence of agricultural chemicals in these lands. Because some of the old, persistent agricultural chemicals have demonstrated estrogenic potential, this is a plausible mechanism of endocrine and reproductive dysfunction.

Experimental Objectives and Hypothesis

The objective of this set of studies was to prepare gene-specific cDNA probes to characterize the coordinate regulation of ER and Vtg mRNA relative to plasma Vtg in largemouth bass (LMB). Another priority was to find other estrogen-regulated genes to complement the understanding of coordinate gene expression in fish. The primary hypothesis tested was that E_2 would induce a primary and delayed primary hepatic transcriptional responses over time for ER and Vtg mRNAs, respectively. This acute exposure to E_2 would also elicit a cascade of differential responses at the mRNA level through up- and down- regulation of known and unknown genes that could be identified by differential display.

Materials and Methods

Fish Collection and Maintenance

Largemouth bass (*Micropterus salmoides*) were purchased from American Sportfish Hatchery (Montgomery, Alabama). They were either maintained at the Aquatic Toxicology Facility Lab at the University of Florida under the direction of Dr. Evan Gallagher or at the United States Geological Survey- Caribbean Science Center under the direction of Dr. Timothy Gross. All experiments were designed and conducted under my direction. Fish were acclimated in aerated 104 to 250 gallon fiberglass tanks (Figure 2-3A and 2-3B), under constant conditions of 21 +/-2° C for a minimum of one week prior to exposures. Dissolved oxygen, total ammonia content, and pH were monitored, but did not vary significantly during acclimation and exposure periods. The fish were exposed to ambient light concentrations, and fed Purina Aquamax 5D05 fish feed (St. Louis, MO)

once a day. Many lab personnel assisted with the sample collection (Figure 2-3C and 2-3D).

Experimental Exposures

All three experiments were single acute exposures administered by intraperitoneal injection. In the first experiment, adult (> 1 year old) male LMB were injected with ~2 mg 17β-estradiol (E_2)/Kg dissolved in dimethyl sulfoxide (DMSO). The fish were returned to the tank, and sampled at 0, 6, 12, 24, and 48 h post injection. Separate control fish were injected with DMSO or not injected at all. These fish were sampled in July of 1998 and had low to no observable spermatogenic activity (Figure 2-4A).

In the second experiment, adult male LMB were injected with varying doses of E_2 or estrogen mimics, followed by sample collection 48 h later. Chemicals and doses used were as follows: E_2 (0.0005, 0.005, 0.05, 0.5, 5.0 mg/Kg), ethinylestradiol (EE_2) (0.005, 0.05. 0.5 mg/Kg), nonylphenol (NP) (0.05, 0.5, 5.0 mg/Kg), methoxychlor (MXC) (0.05, 0.5, 5.0 mg/Kg) or *o,p'*-dichlorodiphenyl-trichloroethane (*o,p'*-DDT) (0.05, 0.5, 5.0 mg/Kg). All chemicals were dissolved and diluted in DMSO. Control fish were injected with DMSO or not injected at all. NP, MXC, and *o,p'*-DDT were purchased from ChemService (West Chester, PA). These fish were sampled in November, 1999 and had low to moderate spermatogenic activity (Figure 2-4B).

In the last experiment, 100 juvenile LMB (< 1 year old) were injected with a ~2 mg E_2/Kg. The fish were returned to the tank, and sampled at 0.25, 1, 2, 4, 7, 14, or 21 days post injection. Separate control fish were injected with DMSO or not injected at all. Although these fish were less than one year old, the gonads of these fish were developing at the time of sample collection. Following histological examination of the gonads, only

fish exhibiting male characteristics were analyzed (Figure 2-4). These fish were sampled in January, 2001 and had moderate to high spermatogenic activity (Figure 2-4C).

Blood and livers were collected from all experimental and control fish. Blood was drawn using a 1cc tuberculin syringe with 20 guage needle. Blood was stored in a heparinized vessel with aprotinin at 4°C until separation. After centrifugation for 20 min at 4800 x g the plasma was aspirated and stored in aliquots at –70°C until analyzed. One aliquot of the plasma was sent to the lab of Dr. Tim Gross, where steroid analyses by radioimmunoassay were conducted by his staff. The liver was excised, then immediately flash-frozen in liquid nitrogen and stored at –70°C until analysis. Gonads were collected from all fish and preserved in 10% buffered formalin until paraffin-embedding and sectioning for histological examination for sex determination. Paraffin embedding, tissue sectioning and H&E staining were performed by the Histology Core facility at the University of Florida.

RNA Isolation

RNA was isolated as described previously by one of two methods (Bowman & Denslow 1999). Briefly, the individual liver tissues were processed for RNA using the acid phenol guanidinium-isothiocyanate (Chomczynski & Sacchi 1987), or using RNeasy kits from Qiagen (Valencia, CA). The samples were treated with Proteinase-K, then measured at 260 and 280 nm using a spectrophotometer. RNA samples used in differential display were also DNase treated. The 260 nm measurement was used to estimate the concentration of total RNA recovered from the isolation. The 260/280 ratio, as well as a 1% agarose-formaldehyde gel stained with ethidium bromide, were used to verify the quality of the RNA in each sample.

Estrogen Receptor and Vitellogenin RT-PCR

Because of the high homology of ER across vertebrate species, nucleotide specific primers were designed based on sequence homologies in the hormone binding domain of human, chicken, frog, and trout ER α. Vitellogenin, however, had very disparate nucleotide similarities, so degenerate primers were designed based on protein sequence homologies in the 3'-end of the Vtg sequences available at that time. The ER α primer sequences were: upper –TCA CCA TGA TGA CCC TGC TCA; lower –TGC TCC ATG CCT TTG TTG CTC. The sequences of the Vtg primers were: upper -CAR GTN YTN GCN CAR GAY TG; lower -GCA YTC NSW NGC RTC NCK RC. Both sets of primers were designed used Oligo 5.0 (Cascade, CO).

Oligo-dT primers, dNTPs, 5X transcription buffer (Life Technologies; Rockville, MD), and Superscript II (Life Technologies) were used to reverse transcribe 2 μg total RNA. Specific ER (10 pmol/μl) or degenerate Vtg primers (80 pmol/μl) in 10X reaction buffer with $MgCl_2$ (Perkin Elmer; Foster City, CA), dNTPs, AmpliTaq (Perkin-Elmer), and 2 μl of cDNA from the reverse transcription reaction were used to amplify portions of the ER or Vtg gene by the polymerase chain reaction (PCR). The PCR conditions for both genes were: hold at 80°C for 3 min; hold at 94°C for 3 min; 35 cycles of 94°C for 45sec, 52°C for 90sec, 72°C for 45sec; hold at 72°C for 10 min; and hold at 4°C for one hour. The PCR products were analyzed and purified by 1.2% agarose gel electrophoresis (Figure 2-5).

Cloning and Sequencing of Isolated cDNAs

The single bands corresponding to the amplified ER or Vtg cDNA were extracted from the gel and purified using Qiaquick gel extraction spin columns (Qiagen). The

purified PCR products were ligated into a pGEM-T Easy vector (Promega; Madison, WI) (Figure 2-6) and transformed into *E.coli* (DH5α). Plasmid clones containing the amplified fragments were picked randomly and purified for sequence determination and probe preparation. Using an ABI PRISM Dye Terminator Cycle Sequencing Kit (Perkin Elmer) and M13 primers from the DNA Synthesis Core Facility (Biotechnology Program, University of Florida), sequencing reactions were performed on the isolated plasmid preparations, and submitted to the DNA Sequencing Core Facility at the University of Florida for sequence determination. BLAST (Altschul et al. 1997) and multiple sequence alignment programs (Corpet, 1988) were used to analyze the results.

mRNA Quantification

Complementary DNA probes were made using the cloned ER or Vtg fragments. Templates for each probe were cut from the plasmid vector using EcoRI (Figure 2-6). The digestion reactions were purified by gel electrophoresis and extracted using Qiaquick spin columns (Qiagen). These templates were used for the synthesis of [α-^{32}P]-labeled cDNA probes using a Strip-EZ DNA Kit (Ambion; Austin, TX) according to manufacturer's instructions, and purified using TE-Midi SELECT-D, G50 spin columns (5 Prime-3 Prime, Inc.; Boulder, CO). A β-actin cDNA probe (GenBank Accession #) was also prepared as described for ER.

ER and Vtg mRNA were quantified as described previously (Bowman & Denslow 1999, Bowman et al. 2000). Briefly, for Northern blot analysis, 12 μg of total LMB liver RNA was denatured and separated on a 1% agarose-formaldehyde gel. The RNA was transferred to a nylon membrane (Biodyne B, Life Technologies) using downward capillary action, followed by UV-crosslinking using a Stratalinker 1800 (Stratagene; La Jolla, CA). Nylon membranes were stained with methylene blue to verify

successful transfer and even loading of the samples (Herrin & Schmidt 1988). The membranes were pre-hybridized in a glass cylinder using a Techne Hybridiser oven with ExpressHyb hybridization buffer (Clontech; Palo Alto, CA) for 30 min at 68°C. The membranes were then incubated at 68°C for one hour in fresh hybridization buffer containing approximately 3 x 10^5 dpm ER, Vtg, ERp72 or β-actin probe/mL. Nylon membranes were then washed twice with 2X SSC, 0.1% SDS for 20 min at 25°C, then twice with 0.1X SSC, 0.1% SDS for 30 min at 60°C or 68°C. The nylons were then wrapped in saran wrap and exposed to BioMax MR X-ray film (Eastman Kodak; Rochester, NY) for visualization, and were exposed to a phosphorscreen for image quantification using a PhosphorImager (Molecular Dynamics, Inc.; Sunnyvale, CA).

For the quantification of ER and Vtg mRNA, sense ER and Vtg cRNA was transcribed *in vitro* from the vector (Figure 2-6) using a Megascript Kit (Ambion) according to the manufacturer's suggestions. Sense ER and Vtg cRNA was analyzed by gel electrophoresis and quantified by spectrophotometry. Concentrations from 0.01 ng to 5 μg Vtg cRNA serving as standards were denatured and loaded onto a Biodyne B nylon membrane using a slot blot apparatus (Schleicher and Schuell; Keene, NH). Twelve μg of sample were denatured in denaturing buffer (containing 20X SSC, formamide, and formaldehyde) and loaded into slots on the same membrane. The nylon membrane was stained with methlyene blue and hybridized as described above for Northern blots (Figure 2-7A and 2-7B).

To standardize gene expression across individuals, Northern and slot blots were stripped and re-probed with β-actin. All ER and Vtg mRNA sample values obtained were corrected individually to β-actin levels. Data was collected using a PhosphorImager

as described above. Actual ER and Vtg mRNA values reported were calculated from a standard curve generated from the synthesized cRNA standards (Figure 2-7C).

Differential Display RT-PCR

Differential display-reverse transcription polymerase chain reaction (RT PCR) was performed with the RNAimage mRNA Differential Display system (GenHunter; Nashville, TN) using one-base anchored oligo-dT primers (Liang et al. 1994). DNase-treated total RNA (0.2 μg), isolated from control or treated LMB livers was reverse transcribed using 0.2 μM of anchor primer and 100 U MMLV reverse transcriptase in a total volume of 20 μL as described by the manufacturer. For each condition, we used three separate liver samples to distinguish false positives. PCR reactions (20 μL) were performed following the RNAimage protocol and included one-tenth volume of the reverse transcription reaction, 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 μM anchor primer and arbitrary primer, 2 μM dNTPs, 2.5 μCi-[^{33}P]-dATP (2000 to 4000 Ci/mmol), and 1 U AmpliTaq DNA polymerase. Primer pairs used are indicated on each figure. After an initial denaturing step of 94°C for 5 min, 40 PCR cycles were run with the following conditions: 94°C for 15 sec; 40°C for 2 min; 72°C for 30 sec; followed by a 10 minute 72°C extension step. Aliquots of each PCR reaction were heated for 3 min at 80°C with DNA sequencing loading dye and separated by electrophoresis on 5% denaturing Long Ranger gels. The gels were dried under vacuum at 80°C and exposed to Biomax MR X-ray film for 18 to 48 h.

Bands of interest were located on the gel, cut out, and then soaked in 100 μL of ddH_2O for 10 min, followed by boiling for 20 min to elute the DNA. A fraction of the material was used as template with the same primer pairs to reamplify the DNA. A 40 μL reamplification reaction was prepared for each cDNA with arbitrary primer 10, 21, or

23 (2 μM) and anchor G- or C-T_{11}primer (2 μM). The PCR conditions were set up as follows: 80°C for 3 min → 95°C for 2 min → 40 cycles of 94°C for 15 sec, 40°C for 2 min, 72°C for 30 sec → 72°C for 10 min → 4°C soak. Twenty microliters of the PCR samples were run on a 1% agarose gel and stained with ethidium bromide. If the size of the reamplified product was correct, the cDNA band was gel purified, cloned and sequenced as described above.

Protein Analysis

The presence of Vtg in serum samples was verified by electrophoresis using Tris-tricine gels and Western blot analysis as described previously (Denslow et al. 1997a). All plasma samples were diluted 50-fold in 1X Laemmli sample buffer and 10 μL were applied to separate wells of 7.5 % polyacrylamide gels (0.2 μL plasma per well). Samples of purified LMB Vtg (0.1 and 0.5 μg) were included in separate wells as positive controls. Multimark molecular weight markers (Novex; San Diego, CA) were used to calibrate the gels. The gels was electroblotted to PVDF membranes (Immobilon-P-Millipore; Bedford, MA) in 10 mM MES (morpholino ethane sulfonic acid), 10% methanol, 0.01% SDS, pH 6.0, overnight at 20 V and 4°C. For Western blot detection, the membrane was blocked with 5% nonfat dry milk in TBSTZ (10 mM Tris, 150 mM NaCl, 0.05% Tween, 0.02% sodium azide, pH 7.2) at 25°C for 2 h. The blot was incubated with primary monoclonal antibody 1C8 HL 1364 in blocking solution, developed with a secondary goat-anti-mouse alkaline phosphatase-linked antibody (Pierce; Rockford, IL) and developed with bromochloroindoyl phosphate/nitro blue tetrazolium as described previously (Denslow et al. 1997b).

A direct ELISA (enzyme-linked immunosorbent assay) was used to quantify plasma Vtg as described previously (Denslow et al. 1999b, Folmar et al. 1996, Folmar et al. 2000) using avidin-biotin complex reagents (Pierce). The overall sensitivity of the ELISA used in this study is 2 μg/mL for plasma Vtg. The assay itself is more sensitive by a factor of 200 when pure Vtg is used. We are able to measure 0.5 ng Vtg per ELISA well in a volume of 50 μL. The lower sensitivity for plasma samples is due to the requirement to dilute plasma 1:200 to eliminate interferences. The linear portion of the standard curve extends from 0.01 to 0.8 μg/mL purified Vtg. All of our standard curves are prepared with added control male plasma, diluted to the same concentration as the samples, so that the samples and standards have the same composition. Two different standard curves were performed with male plasma diluted to 1:200 (for the samples containing the least amount of Vtg) and 1:10,000 (for the highly induced samples). On occasion a sample will have a Vtg concentration that is outside these standard curves. In that event, we repeat the assay with the appropriate standard curve. The primary antibody used was 3G2 109AB raised against striped bass Vtg. The plate coated with samples and standards was incubated with the monoclonal antibody overnight at 4°C in a humidified chamber. For the rest of the assay the following reagents from Pierce were used: goat-anti-mouse IgG (H&L), biotin, and the streptavidin alkaline phosphatase conjugate. After washing the plate, Vtg was quantified colorimetrically with the alkaline phosphatase substrate, *p*-nitro phenyl phosphate (in carbonate buffer with 2 mM $MgCl_2$, pH 9.6) at 405 nm in an ELISA plate reader. All samples and standards were run in triplicate. The coefficients of variation and correlation coefficients for this assay were <10% and > 0.95% respectively.

Results

Estrogen Receptor and Vitellogenin Sequencing and Characterization

Because the cloned LMB ER and Vtg fragments were initially obtained by PCR, five independent clones in both orientations (both strands) were sequenced. Sequence analysis revealed distinct sequences for the two genes. A BLAST search (Altschul et al. 1997) of each showed specific homology to most of the ER α or Vtg sequences in the database. As shown in Figure 2-8, the LMB ER α sequence (Genbank accession number AF253062) was aligned with ER α sequences from human to fish. The LMB Vtg sequence (Genbank accession number AF169287) had greater than 70% identity to the various Vtg sequences (Figure 2-9). The Vtg sequence is not as well conserved relative to the ER so there is less homology across species for this gene.

In order to verify that the ER and Vtg mRNAs were induced by E_2 as expected, ER and Vtg gene expression were analyzed by Northern blot. Using random-primed cDNA probes from these cloned cDNAs by Northern both genes were up regulated in response to estrogen (Figure 2-10A). ER mRNA in LMB is approximately 3.5 kb in length. The ER cDNA probe used in these studies was shown to be specific for ER α without crossreactivity to ER β in LMB (unpublished observations). Our Vtg probe detected 3 distinct bands at 5.0, 3.3, and 1.7 kb for this mRNA in exposed samples only (Figure 2-10A). Crossreactivity of the Vtg probe with 28S (4.0 kb) and 18S (1.9 kb) rRNA does not account for the smaller two Vtg bands since control samples do not present the bands (Figure 2-10A and 2-10C). All lanes were loaded with equal amounts of total RNA. Furthermore, the calculated sizes of the Vtg bands are different than the rRNA bands. Following hybridization with ER α and Vtg, a β-actin probe showed a

steady amount of β-actin mRNA (2.1 kb) across both control and exposed sets of RNA samples (Figure 2-10B). RNA size markers (Promega), 28S rRNA (4.0 kb), and 18S rRNA (1.9 kb) were used to calibrate the blots when determining the respective ER and Vtg mRNA sizes.

Regulation of mRNAs in Adult Largemouth Bass 48 Hours Post Injection

Following the injection of E_2 (2 mg/Kg), plasma steroids were measured at 0, 6, 12, 24, and 48 h (Figure 2-11). Plasma testosterone stayed below 500 pg/mL for the duration of the experiment and was not significantly different from controls at any of the timepoints. As expected, plasma estrogen increased approximately 6-fold over controls by 6 h post injection. The average amount measured at 6 h, 2,806 pg/mL, did not vary significantly out to 48 h post injection. The average body weight of these adult fish were 165 g.

The expression of ER and Vtg mRNA was analyzed by both Northern blot (Figure 2-12A and 2-12B) and slot blot (Figure 2-12D and 2-12E). Since slot blot values were obtained using a standard curve and are corrected for individual β-actin values, these were considered more quantitative. Both methods demonstrated the same trends, ER and Vtg mRNAs were detectable by 6 h and continued to increase for the duration of the experiment (Figure 2-12). There was a distinct increase in ER mRNA by 12 h, and by 48 h there was approximately 3.68 pg/μg total RNA. Following injection, Vtg mRNA nearly tripled by 12 h and had greater than a 10-fold increase by 48 h (approximately 533 pg/μg total RNA). Estrogen receptor mRNA levels appeared to increase slightly faster than Vtg mRNA, particularly evident by 12 h post injection (Figure 2-12A and 2-12B). Although not seen by Northern, the slot blot data detected a low basal level of Vtg mRNA (0.04 ng/μg total RNA) in control fish.

Plasma Vtg, analyzed by both Western blot and ELISA, demonstrated the expected timed induction relative to Vtg mRNA. In Western blots, two bands corresponding to 160 and 180 kDa were seen within 6 h post injection. The 160 kDa band appeared to be more highly expressed and more consistent at 12 and 24 h. By forty-eight hours, both bands plus some intermediate size fragments, presumably breakdown products of the 180 kDa band, are visible.

Determination of plasma Vtg by ELISA is more quantitative than by Western blot. Standard curves using purified LMB Vtg coupled with control male plasma were used to generate standard curves. Following injection, Vtg was measurably induced by 6 h, then increased up to 1 mg/mL at 24 h. By forty-eight hours Vtg protein accumulated in the plasma to approximately 4 mg/mL (Figure 2-13).

In order to look for other genes that might also be regulated by estrogens, differential message display RT-PCR was used over time following acute exposure (Figures 2-14 and 2-15). Equal amounts of control or exposed samples were loaded on the gel for side-by-side comparison. As expected some mRNAs were induced by estradiol in the same time frame as Vtg. Others were induced earlier; some even returning to baseline by 48 h post injection. PCR-amplified products using primer pairs G-23, C-1, and G-10 were a good illustration of some differentially regulated genes. The arrows on the left of Figure 2-14 show examples of specific mRNAs that are down regulated by E_2. As shown by the arrows on the right side of the gel, some cDNAs were even transiently regulated, up by 12 h and down by 48 h (Figure 2-14). As would be expected, the regulation of some cDNAs did not change over time following E_2-exposure as indicated by the arrows on the left in Figure 2-15. Arrows on the right illustrate that some cDNAs were up regulated post injection (Figure 2-15). Twelve of the bands shown

were cut out of the gel, cloned, and sequenced in an attempt to identify what mRNA they represented. The sizes of the amplified products were from 75 to 560 bp in length. Since all the cDNAs are amplified from their 3'-ends, sequence information often does not get into the coding region making gene identification difficult.

One of the few bands identified so far is Vtg (Figure 2-15). The other is a protein disulfide isomerase-related protein, known as ERp72. This identity was discovered following BLAST analysis and aligning the predicted amino acid sequence with that of known human, rat, and mouse (Figure 2-16A). Verification of this E_2 up regulation over time by Northern is shown in Figure 2-16B and 2-16D. Largemouth bass ERp72 mRNA appears to be the first protein disulfide isomerase-related protein observed in teleosts and is approximately 2.6 kb in size. Following estradiol-injection, LMB ERp72 mRNA reached maximum levels (approximately 4-fold) by 24 h, and rapidly decreased by 48 h, but did not return to near basal levels until 21 days post injection (Figure 2-16D). This pattern of induction over the first 48 h is different from that observed on the DD-RTPCR gel (Figure 2-15), demonstrating the importance of validating all observed responses. One possible reason for this discrepancy is that the clone sequenced from the band on the gel is only one of the many cDNAs that may be present at that particular size, and may not represent the higher intensity band seen on the gel (Figure 2-15).

Plasma Vitellogenin Dose Response

In the second experiment plasma sex steroids and Vtg were measured from all male fish (Figure 2-17 and 2-18). These measurements were taken forty-eight hours following the injection of several different doses and chemicals. Forty-eight hours following the 5 mg E_2/Kg dose, plasma E_2 increased approximately 4-fold. Although 0.05 and 0.5 mg E_2/Kg did not increase plasma E_2, the two lowest doses (0.0005 and

0.005 mg/Kg) increased plasma E_2 levels significantly over controls. Nonylphenol increased plasma E_2 in a dose dependent manner up to 600 pg/mL (Figure 2-17A). Both of these higher doses of NP increased plasma E_2 significantly over controls ($P < 0.05$). All doses of MXC significantly increased plasma E_2 up to 500 pg/mL by 48 h post injection ($P > 0.05$). Plasma estradiol levels were not affected by EE_2 or DDT. Plasma 11-ketotestosterone (11-KT) was highly variable but was not statistically different from controls between any of the chemicals or dose, with the exception of the 0.05 and 5 mg/Kg of E_2 (Figure 2-17B). Plasma steroids in placebo-treated animals (DMSO) taken at 48 h did not differ from uninjected control fish (data not shown).

Estradiol induced plasma Vtg in a dose-dependent manner up to approximately 9 mg/mL by 48 h post injection (Figure 2-18A). The two lowest doses of E_2 (0.0005 and 0.005 mg/Kg) did not appear to induce plasma Vtg. Therefore the lowest dose with an observed effect on Vtg induction was 0.05 mg E_2/Kg. Acute ethinylestradiol-exposure also elicited a dose dependent increase in plasma Vtg (Figure 2-18B). All doses of EE_2 tested increased plasma Vtg; the lowest dose induced plasma Vtg accumulation up to 4 mg/mL. As shown in Table 2-1, none of the doses of NP, MXC, or DDT induced plasma Vtg over the limit of detection by 48 h post injection.

mRNA Characterization After Acute Exposure in Juvenile Largemouth Bass

In the third experiment, samples were collected at 0.25, 1, 2, 4, 7, 14, and 21 days following the injection of ~2 mg estradiol/Kg. The average body weight of these fish was 42.2 g. Sex steroids of these fish were not analyzed because of insufficient quantities of plasma. Analysis of ER and Vtg mRNA by Northern blot was for qualitative purposes (Figure 2-19A and 2-19B). Since slot blot values were obtained using a standard curve and were corrected for individual β-actin values, these were

considered more quantitative. Both methods show the same pattern (Figure 2-19). Both ER and Vtg mRNA were up regulated by 6 h (time point 0.25). The pattern of induction out to 2 days was similar to that observed in the earlier experiment with the adult LMB, quickly increasing to maximal levels. By day 7 and for the duration of the three weeks, both ER and Vtg mRNA returned to levels observed for the 6 hour time point. In these juvenile fish, ER mRNA was maximally induced by 2 days post injection with ~3.5 pg/μg total RNA. Vitellogenin mRNA was induced up to ~138 pg/μg total RNA, approximately 30-fold over the basal level. Estrogen receptor mRNA appeared to increase faster over time than Vtg in the initial 6 h, and by four days return to minimal plateau level quicker as well (Figure 2-19). Although not apparent by Northern, the slot blot data detected a low basal level of ER mRNA (1 pg/μg total RNA) and of Vtg mRNA (0.004 ng/μg total RNA) in control fish.

Plasma Vtg, analyzed by ELISA, demonstrated the expected timed induction relative to Vtg mRNA. Following injection, Vtg was measurably induced by 6 h, then up to 0.48 mg/mL at 24 h, and by 2 days it accumulated in the plasma to approximately 1.4 mg/mL (Figure 2-20). Rather surprisingly, by 4 days plasma Vtg levels had already begun to decrease, going down to 0.76 mg/mL. At 21 days it was still detectable in the plasma at levels around 0.13 mg/mL.

Discussion

Unfortunately, the same features that make LMB a model sentinel species also make it difficult to study in the laboratory. Specifically, its large size and dominant presence in freshwater lakes make it a representative species in these ecosystems. Its large size and apparent sensitivity to environmental perturbation also make it hard to

raise and maintain in tanks, relative to the care of smaller laboratory model fish species. LMB reproduction is synchronized over the course of each year. Thus, it is useful for monitoring reproductive outcomes, but makes it difficult for short-term laboratory studies because the outcome my depend on the time of the year. To monitor this possible confounding effect, the fish from each experiment were examined by histology to determine reproductive status during each experiment (Figure 2-4). It was not expected that the exposure end points measured in the male fish from all these experiments would be affected by reproductive status.

Largemouth Bass Estrogen Receptor and Vitellogenin

To begin these studies, the first step was to clone portions of the ER and Vtg gene from LMB. Since the amino acid sequences of ER are similar across species; human, rat, frog, and trout ER sequences were used to design primers. Because the DNA-binding domain is so similar across all nuclear receptors, the primers were made to a homologous region specific for only ER α using the hormone-binding domain. Based on the sequences used to design these primers the predicted size of the amplified product would be approximately 550 bp. Using control and estrogen-treated LMB a band corresponding to this size was obtained by PCR (Figure 2-5A).

The predicted amino acid sequence from the cloned LMB ER cDNA fragment lined up with greater than 80% identity with the published sequences for rainbow trout (Pakdel et al. 1990), gilthead seabream (Socorro et al. 2000), and tilapia (Tan et al. 1996). It also has significant similarity (> 65%) to the human (Greene et al. 1986), rat (Koike et al. 1987), and mouse (White et al. 1987) ER α (Figure 2-8). At the time this sequence was cloned, there was only one estrogen receptor identified in fish. Therefore all the work presented in this dissertation is of ER α only. In recent years an ER β was

identified in teleosts (Tchoudakova et al. 1999, Xia et al. 1999). Just last year there was even a report of a third isotype, ERγ, in Atlantic croaker (Hawkins et al. 2000). The unique tissue and ligand specificities of all three isoforms have implications for the research presented in this dissertation and are areas of future study in the Denslow laboratory. Currently both ER β and γ isoforms have been cloned and sequenced for LMB (Sabo-Attwood et al. in preparation).

The amino acid sequence of vitellogenin is poorly conserved across oviparous species. When the available sequences in Genbank are aligned, sequence similarity exists only in short segments of amino acids along the full length of the protein with the exception of the phosvitin region, where it is greater than 50% phosphoserine. When designing our Vtg primers, a region close to the C-terminus of the protein was selected where there was the most apparent conservation among sequences for trout, sturgeon, lamprey, and two mummichog sequences. These primers are generally useful for cloning Vtg mRNA segments from a large variety of fish including largemouth bass, Genbank accession number AF169287 (Bowman & Denslow 1999), gilthead seabream, *Sparus aurata* (Funkenstein et al. 2000), bluegill, tilapia, koi, trout, and pinfish (Bowman and Denslow, unpublished).

The LMB Vtg cDNA fragment cloned was from the 3' region of the very large and heterogeneous Vtg mRNA (5 kb estimated). The predicted amino acid sequence for this LMB Vtg cDNA fragment had greater than 70% homology to the other published fish sequences, specifically to the mummichog (LaFleur et al. 1995a), tilapia (in Genbank only, accession number AF017250), and trout (Mouchel et al. 1996). Chicken (van het Schip et al. 1987), and frog (Gerber-Huber et al. 1987) Vtg sequences had approximately 40% homology (Figure 2-9). There is evidence to suggest the presence of two Vtgs in

teleosts (Bowman et al. 2000, LaFleur et al. 1995b, Matsubara et al. 1999). The two Vtgs in flounder have been reported to have distinct functions in egg maturation (Matsubara et al. 1999). In the past month the Denslow lab isolated a fragment of a second Vtg in LMB.

Since ER and Vtg mRNAs had not been previously characterized in LMB, total RNA from liver was subjected to Northern blot hybridization using gene-specific probes. Northern blot analysis of ER mRNA revealed one band of approximately 3.5 kb only in the E_2-treated fish (Figure 2-10A). This is the same size as the primary liver ER mRNA (3.5 kb) reported in E_2-treated rainbow trout (Pakdel et al. 1989). Additional transcript sizes of ER have been reported in trout liver (4.5 kb) (Pakdel et al. 1989), trout pituitary (1.4 kb) (Pakdel et al. 1990), and catfish liver (several ER α sizes between 1.5 and 10 kb)(Patino et al. 2000), but these were not evident in LMB liver using this cDNA probe.

Northern blot analysis of LMB Vtg mRNA revealed three bands of 5.0, 3.3, and 1.7 kb in length in E_2-exposed fish only (Figure 2-10A). The most intense band at 5.0 kb is of sufficient size to contain the entire predicted coding sequence for LMB Vtg (160 to 180 kDa) (Figure 2-13A). Largemouth bass Vtg appears similar in size to that reported in sheepshead minnow (5.0 kb) (Bowman et al. 2000), but smaller than the reported size for rainbow trout (6.6 kb) (Le Guellec et al. 1988) and tilapia (6.5 kb) (Lim et al. 1991). Size calibration techniques, however, may account for some of the apparent species difference in Vtg mRNA sizes. The two minor bands observed in LMB (3.3 and 1.7 kb) are too small to contain the entire predicted coding sequence for Vtg. These smaller bands could be smaller Vtg mRNA transcripts, specific mRNA degradation products, or alternative splice variants. It is possible that they represent highly similar mRNAs that contain sequences that are homologous to our probes. Belonging to the apolipoprotein gene

family (Wahli et al. 1981), Vtg may share significant homology in some regions with other proteins in the family. This is not likely since the bands persisted following very stringent washing at 68°C. Other studies report similar smaller Vtg transcripts. At least 4 small putative Vtg transcripts at 3.8, 3.1, 1.9, and 1.3 kb have been reported in several fishes from the family Cichlidae (Lee et al. 1992).

Exposure of Adult Largemouth Bass to Estradiol

The first experiment on adult (>1 year old) LMB was conducted to establish a time course of ER and Vtg mRNA induction to E_2 relative to plasma Vtg accumulation. Plasma estradiol levels were about 2500 pg/mL plasma at each of the time points collected: 6, 12, 24, and 48 h post injection (Figure 2-11). Data from a single E_2-injection of fathead minnow (FHM) seemed to result in decreasing levels of plasma E_2 over time, returning to baseline by 48 h (Korte et al. 2000). It is not clear why plasma E_2 did not decline rapidly between 6 to 48 h following injection in LMB, but this may reflect a slower clearance from the plasma in LMB than in fathead minnow. In addition to species variation, different carrier solvents were used in for the two exposures, DMSO (LMB) and ethanol/corn oil (FHM), and that may account for the clearance differences between the two experiments. In that FHM study 70% of the injected E_2 is quickly cleared by 8 h post injection indicating rapid assimilation into the bloodstream (Korte et al. 2000). That initial bolus of E_2 (thought to have occurred in LMB as well) probably led to the increase in ER and Vtg mRNA observed in LMB.

Over the forty-eight hours following the 2 mg/Kg injection in male adult LMB, ER and Vtg mRNA increased to observed maximum amounts at 48 h of 3.68 and 533 pg/μg total RNA respectively. This pattern of Vtg mRNA accumulation over 48 h is supported by studies in rainbow trout (Le Guellec et al. 1988), fathead minnow (Korte et

al. 2000), and sheepshead minnow (Bowman et al. 2000). The increase of both ER and Vtg over 48 h is consistent with another report in rainbow trout (Pakdel et al. 1991). Although the pattern of induction over 48 h is similar, the measured amounts of ER and Vtg mRNA are about 10-fold lower in LMB compared to rainbow trout (Pakdel et al. 1991). According to Figure 2-12, LMB ER mRNA appears to accumulate faster than Vtg mRNA up to 48 h. To better illustrate this, Figure 2-21 replots this raw data over the initial 48 h. Figure 2-21C expresses the mRNA induction as a percent of the maximum response, this demonstrates that ER mRNA increases faster at 12 h than Vtg mRNA. This is even more obvious if the change in mRNA accumulation over time is plotted against time alone (Figure 2-21E). These data are consistent with a primary response for ER mRNA induction followed by a delayed primary response of Vtg mRNA (Figure 2-1) over 24 h as demonstrated previously in rainbow trout (Pakdel et al. 1991).

The accumulation of plasma Vtg was also measured both qualitatively and quantitatively. Western blots using specific monoclonal antibodies show the increase of two bands in exposed samples (Figure 2-13A). It is not clear at this time if the two bands represent distinct Vtg proteins or modifications of the same protein. However, one of our two LMB monoclonal antibodies only detects one band, consistent with two distinct amino acid sequences between the two bands. The use of monoclonal antibodies and species-specific purified protein in the ELISA assay is more sensitive and specific than using polyclonal antibodies with general standards (as discussed by Korte et al. 2000 using fathead minnow). This is because it measures a unique amino acid signature (epitope) on a protein resulting in a one antibody per protein quantification. By both Western and ELISA, plasma Vtg appeared to mimic the increase in Vtg mRNA over time, without much delay in time (Figure 2-13). This was unexpected since other studies

have shown the accumulation of plasma Vtg to be delayed relative to Vtg mRNA (Bowman et al. 2000, Korte et al. 2000).

By characterizing the *in vivo* increase in both Vtg mRNA and protein, it is possible to monitor different levels of the ER-mediated pathway. Together these represent activation of transcription in the nucleus to mature protein secreted into the plasma of the animal. It has been mentioned previously that Vtg mRNA and protein serve as distinct biomarkers of estrogen exposure in male oviparous vertebrates over time (Bowman et al. 2000, Korte et al. 2000).

Plasma Steroid and Vitellogenin Dose Response to Estrogens

The purpose of this experiment was to test the sensitivity of adult LMB to various estrogens 48 h following acute exposure. Specifically, a full dose response was tested for estradiol, while smaller ranges of dose were examined for ethinylestradiol (EE_2), nonylphenol (NP), methoxychlor (MXC), and *o,p'*-dichlorodiphenyl-trichloroethane (DDT). The apparent impact of these chemicals on plasma sex steroids 48 h post injection was minimal with the following exceptions. Forty-eight hours following the 5 mg E_2/Kg dose, plasma 11-KT approximately doubled and plasma E_2 increased approximately 4-fold (Figure 2-17), both effects were significant over controls ($P < 0.05$). This effect of the highest dose of E_2 (5 mg/Kg) is interesting, as it suggests that lower levels of injected E_2 are quickly metabolized and cleared from the fish in order to maintain E_2 homeostasis. The increased levels of plasma E_2 at the lower doses (0.0005 and 0.005 mg/Kg) but not the intermediate doses (0.05 and 0.5 mg/Kg) at 48 h is difficult to explain, but could be an artifact of low sample numbers (n=5) per group. These higher levels of E_2 may overwhelm the system resulting in a change in the steady state levels of the hormone, which could have profound biological effects. The increased levels of 11-

KT, most noticeable at the highest dose of E_2 (Figure 2-17B) is also interesting since the previous experiment showed no change in plasma testosterone at 48 h following 2 mg E_2/Kg-injection. The reason for this difference in testosterone and 11-KT levels 48 h post E_2-injection is not clear at this time. This effect of E_2 on 11-KT does suggest that exogenous estrogen exposure is capable of affecting the levels of other plasma sex steroids, perhaps through feedback control of steroid biosynthesis.

None of the xenoestrogens tested (EE_2, NP, MXC, or DDT) affected plasma 11-KT levels at 48 h post injection in LMB. It was interesting that plasma E_2 was increased by NP in a dose dependent manner up to 600 pg/mL (Figure 2-17A), with both the higher doses significantly higher than controls ($P < 0.05$). This phenomenon is not easily explained, since NP is a weak ER agonist (Petit et al. 1997) acting much farther downstream than E_2 synthesis or metabolism. These data support the possibility that the biological activity of NP is not just at the level of the ER, but may also affect steroidogenesis or steroid metabolism. This increase in plasma E_2 following NP exposure is consistent with a similar observation in fathead minnow (Giesy et al. 2000), but conflicting with a more recent study by the same lab using nonylphenol ethoxylates (Nichols et al. 2001). This would indicate that this effect on plasma E_2 is unique to the final breakdown product (NP) of nonylphenol polyethoxylates. Another study using flounder exposed to octylphenol (2 mg/Kg), another alkylphenol, increased plasma E_2 as well (Mills et al. 2001). However, a study in Atlantic salmon reported that 5 mg/Kg NP actually decreased plasma E_2 2 weeks following injection (Arukwe et al. 1997). These data would indicate that this apparent increase in plasma E_2 by NP may be dependent on the species and the time of measurement.

All doses of MXC appeared to increase plasma E_2 compared to controls ($P < 0.05$) by 48 h post injection (Figure 2-17A). This increase in plasma E_2 by MXC was also unexpected, but it was not dose dependent at the doses and time selected. Therefore it seems to be impacting steroidogenesis or steroid metabolism in a manner unique from NP. There is evidence that NP and MXC induces cytochrome P450s, specifically CYP3A (Lee et al. 1996a, Li et al. 1995). NP even seems to decrease CYP1A mRNA levels and inhibit 7-ethoxyresorufin O-deethylase activity (Lee et al. 1996b). How MXC appears to induce CYP2B and 3A has been investigated (Li & Kupfer 1998), but little has been done to determine the physiological effects of this metabolic activation. There is also evidence that MXC inhibits the enzymatic activity of CYP3A, probably inactivating the induced enzyme (Li et al. 1993). There is also evidence that MXC inhibits, but not inactivates, CYP2A1, 2B1/B2, and 2C11 (Li et al. 1993). Specifically the inhibition of 2-hydroxylation of E_2 by MXC (Li et al. 1993) may result in the increased levels of plasma E_2 in this study. More studies into this possible mechanism are obviously warranted, especially since NP and MXC appear to be affecting plasma E_2 levels differently. Either way, the accumulation of plasma E_2 is probably a function of altered metabolism by these xenoestrogens. Plasma estradiol levels were not affected by any doses of EE_2 or DDT, even though DDT is known to induce CYP3A as well (Li et al. 1995). Doses of 30 to 120 mg/Kg DDT have been shown to not effect plasma E_2 at 4 to 8 weeks following exposure in flounder (Mills et al. 2001).

As illustrated in Figure 2-18A, E_2-induced plasma Vtg in a dose dependent manner up to approximately 9 mg/mL by 48 h post injection of adult LMB. The accumulation of Vtg obtained at 48 h post injection for the 0.5 and 5 mg E_2/Kg doses is comparable to the levels obtained from the 2 mg/Kg dose tested previously in adult LMB

(4.5 mg/mL). The 0.05 mg/Kg was the lowest dose of E_2 that led to Vtg accumulation by 48 h post injection. The only other chemical that significantly induced plasma Vtg by 48 h was EE_2 (Figure 2-18B). Surprisingly the lowest dose of EE_2 (0.005 mg/Kg) induced plasma Vtg accumulation up to 4 mg/mL. This data demonstrates that EE_2 is much more potent than estradiol in LMB, which is consistent with Vtg induction (Nimrod & Benson 1996) and binding data (Nimrod & Benson 1997) reported in catfish. The higher doses of EE_2 resulted in a dose-dependent increase in plasma Vtg.

As shown in Table 2-1, none of the doses of NP, MXC, or DDT induced plasma Vtg over the limit of detection by 48 h post injection. Despite the relatively low doses evaluated (0.05 to 5 mg/Kg), the lack of plasma Vtg was unexpected since these are suspected xenoestrogens that have been shown to induce Vtg in other systems (Arukwe et al. 1998, Hemmer et al. 2001, Nimrod & Benson 1996, Thorpe et al. 2000). Interestingly, one study in flounder using 30 to 120 mg/Kg DDT did not show an increase in plasma Vtg, albeit at 4 to 8 weeks following exposure (Mills et al. 2001). One explanation is that these weak xenoestrogens may be inducing synthesis of Vtg, but it would not be detectable until after 48 h. A more likely explanation however may be related to the dose tested. In the previous studies the doses tested ranged from 25 to 380 mg/Kg for NP, MXC, and DDT (Arukwe et al. 1998, Nimrod & Benson 1996, Yadetie et al. 1999). In particular, dose response by NP in salmon demonstrated that 5 mg/Kg did not induce plasma Vtg by 1 or 4 days post injection, and 25 mg/Kg did not induce Vtg until 4 days (Yadetie et al. 1999). Therefore, the LMB data are consistent with the potency of these xenoestrogens *in vivo* as presented by other investigators using this route of exposure. According to ER binding studies in catfish, all three of these three xenoestrogens were 1000 times less potent than E_2 (Nimrod & Benson 1997). Therefore

based on the E_2 data in LMB, a dose of > 50 mg/Kg would be needed to induce plasma Vtg. These dose response studies demonstrated that plasma E_2 levels were more sensitive than plasma Vtg when challenged by xenoestrogens at acute doses up to 5 mg/Kg.

Exposure of Juvenile Largemouth Bass to Estradiol

One acute estradiol-injection study in rainbow trout reported that peak levels of Vtg mRNA actually occurred a week following the peak levels of ER mRNA (Pakdel et al. 1991). Since the first time course tested in LMB was only out to 2 days, a longer time course was conducted over 21 days in order to find where the peak expression levels were for these genes in LMB. This study was completed using juvenile LMB (< 1 year old) undergoing their first reproductive cycle as illustrated by histology of their gonads (Figure 2-4C and 2-4D). This gender distinction made it possible to report data for male LMB only.

Northern and slot blot analysis was used to characterize the gene expression pattern of ER and Vtg mRNA following the single E_2-injection (2 mg/mL). Both ER and Vtg mRNAs accumulated to peak levels by 48 h post injection (Figure 2-19). At four days ER mRNA levels had reached a plateau, whereas Vtg mRNA levels were still returning from peak levels. This time point, perhaps more than any other, best distinguishes the temporally distinct pattern in mRNA levels between ER and Vtg mRNA. This difference between the levels of mRNAs is probably a combination of unique mRNA half-lives in addition to the changing rates of synthesis and degradation. By Day 7 through Day 21, both mRNAs had returned to levels initially observed at 6 h post injection. As illustrated in Figure 2-19 the peak amount of ER mRNA produced (3.5 pg/μg total RNA) is only a fraction of the amount of Vtg mRNA (137.8 pg/μg total RNA) induced by the same treatment. Neither mRNA had returned to basal levels by

three weeks post injection. The estradiol levels remaining after 6 h (as shown in Figure 2-11) could be responsible for the maintenance of above normal, but low levels of these mRNAs out to 21 days. Although plasma estradiol was not measured in this particular experiment, previous data from fathead minnow (Korte et al. 2000) would suggest that the initial bolus of E_2 following the injection caused the initial increase in ER and Vtg mRNA observed, but that the lower level of E_2 remaining out to 48 h (Figure 2-11) and perhaps longer could help explain why neither mRNA returned to basal levels by 21 days in LMB.

As shown in Figure 2-19, both ER and Vtg mRNA peaked at 2 days post injection. These data are similar with the previous experiment in adult LMB. This peak of Vtg mRNA induction at 2 days and declining over time is supported by studies using 2 to 5 mg E_2/Kg in rainbow trout (Le Guellec et al. 1988), fathead minnow (Korte et al. 2000), and sheepshead minnow (Bowman et al. 2000). The observation in rainbow trout following single E_2-injection (Pakdel et al. 1991) that reported ER mRNA peak levels (Days 2 to 6) occurring a week prior to Vtg mRNA peak levels (Day 15) is not consistent with these results in LMB or with other Vtg mRNA induction studies cited earlier. It is not clear why the Pakdel study observed such late peak levels of ER or Vtg mRNA. There are many factors that may be responsible for the apparent discrepancy in results between the LMB data and that presented by Pakdel. One possibility is the carrier vehicle used to administer the E_2, Pakdel reports using saline compared to cocoa butter (rainbow trout) (Le Guellec et al. 1988), ethanol/corn oil (fathead minnow) (Korte et al. 2000), triethylene glycol (sheepshead minnow) (Bowman et al. 2000), or in this experiment, dimethylsulfoxide (LMB). The choice of carrier may dramatically affect the uptake, disposition, and rate of release of the chemical over time, especially comparing

saline versus these other carriers. Other factors to consider are the temperature and salt maintenance differences between these freshwater and marine species (rainbow trout at 16°C compared to 25°C for LMB). The reproductive status of the fish is also an important consideration when interpreting and comparing hormone-dependent results between experiments as discussed later, but certainly when evaluating data across species.

The primary hypothesis for these LMB E_2-injection studies was that estradiol would induce a primary and delayed-primary hepatic transcriptional response over time for estrogen receptor and vitellogenin mRNAs respectively. Initially this was based on the observation of temporally distinct (days) peak levels of ER and Vtg mRNA reported in rainbow trout (Pakdel et al. 1991). On closer examination of how a primary and delayed primary response gene was defined, the temporal distinction between the two responses should only be hours, not days (Dean & Sanders 1996). By mechanistic definition, ER and Vtg transcription represent primary and delayed primary responses. What this means is that ER synthesized by the primary response is partially responsible for the transactivation of the Vtg gene (hence a delayed-primary response). Both estradiol-injection time course experiments were designed to test this idea that ER and Vtg mRNA fall into these distinct response categories. As described in detail below, the LMB mRNA data supports the hypothesis that the transcription of ER and Vtg represent primary and delayed primary responses respectively.

The most important time frame for distinguishing these responses are their respective rates of mRNA accumulation compared to the time in which they reach their peak amounts. For both adult and juvenile LMB the ER and Vtg mRNA responses peak at 2 days post injection. Figures 2-21A and 2-21B illustrate the raw data for the

induction of these mRNAs up to their peak levels observed at 2 days. Because both studies were conducted using approximately the same dose, Figure 2-21 also illustrates how age (juvenile vs. adult) can affect the amount of mRNA synthesized. In adult LMB, there appears to be a much lower basal level of ER mRNA, but a higher dynamic range of induction compared to juvenile fish. Conversely adult LMB seem to have higher basal levels and induction capacity of Vtg mRNA than juvenile fish. In addition to age, the differences seen between the two experiments can also be attributed to the reproductive status of the fish. The juvenile fish appeared reproductively active in their first season, but were sampled in January (moderate to high spermatogenic activity) (Figure 2-4C) compared to the adults which were sampled in July (low spermatogenic activity) (Figure 2-4A). It is not known at this time if reproductive status or age may impact the current hepatic mRNA results in LMB, but this is currently being investigated in the lab.

The magnitude of Vtg mRNA induction being so much greater than that of ER mRNA has also been observed previously in rainbow trout (Flouriot et al. 1996, Flouriot et al. 1997, Pakdel et al. 1989, Pakdel et al. 1991). The reason for the different sensitivities and mRNA response to the same E_2-exposure may be the ability of the activated ER complex to bind the gene-specific estrogen response elements (ERE). The ERE in the promoter of the ER gene is thought to be imperfect (perhaps just a half-site), which may help explain the weak induction of this gene compared to the perfect ERE thought to exist in the promoter of the Vtg gene. In addition, the rainbow trout ER gene has a low threshold response to ligand-bound receptor and increasing amounts of ER protein do not affect ER mRNA following E_2-exposure (Flouriot et al. 1997). This is consistent with it being a primary mRNA response.

Post-transcriptional stabilization of the induced mRNAs are also thought to contribute to the levels of ER and Vtg mRNA present in the cell (Flouriot et al. 1996, Shapiro et al. 1989). As reported *in vitro* for rainbow trout using actinomycin D, the half-life of ER mRNA is 4 h and Vtg mRNA is 10 h, however in the presence of E_2 these half-lives were extended to 14 and 29 h respectively (Flouriot et al. 1996). This effect of estrogen on the post-transcriptional stabilization of ER and Vtg mRNA is consistent with other reports in human breast cancer cells (Saceda et al. 1998) and frog (Brock & Shapiro 1983, Dodson & Shapiro 1997). Based on results in LMB, the low level of E_2 remaining in the plasma following injection could be responsible (through mRNA stabilization) for these mRNAs not returning to basal levels by 21 days post injection (Figure 2-19). The end result is that these genes have very specific, but different sensitivities to the concentration of E_2 and ER protein present in the cell at any particular time (Flouriot et al. 1996, Flouriot et al. 1997).

Due to this large difference in the magnitude of the mRNA accumulation between the two genes ER and Vtg mRNA it is difficult to illustrate the distinction between the hypothesized primary and delayed primary responses. Therefore the raw data (Figure 2-21A and 2-21B) is replotted as a percent of the maximum response at 2 days for each mRNA per experiment (Figure 2-21C and 2-21D). This replot illustrates how LMB ER mRNA appears to be up regulated as a higher percent of its peak level earlier than Vtg mRNA (ER mRNA curve is slightly to the left of Vtg mRNA). Perhaps a more appropriate comparison between the accumulations of these genes is the rate of mRNA accumulation using derivative of the raw data (change in mRNA over time/time) as shown in Figure 2-21E and 2-21F. This replot suggests the rate of ER mRNA peaks much earlier (6 to 12 h) than Vtg mRNA (48 h) following acute E_2-exposure.

The results of the analysis in Figure 2-21 is consistent with reports with rainbow trout E_2-exposure where the transcription rate of ER mRNA peaks at 6 to 12 h and peak rates of Vtg mRNA follow at 48 h (Flouriot et al, 1997). Specifically it appears that the ER gene has a high sensitivity, low capacity for E_2-induction, whereas Vtg has a low sensitivity, high capacity for E_2 (Flouriot et al, 1997). Since ER protein accumulation was not measured in LMB during this time, it is difficult to prove that the ER mRNA induced earlier is partially responsible for the short time lag in Vtg mRNA induction. But these LMB data do still support the hypothesis that ER and Vtg mRNA are primary and delayed primary response genes. In fact, an earlier study in rainbow trout demonstrated that ER mRNA does actually appear at least 6 h earlier than Vtg mRNA following single E_2-injection (0.5 mg/Kg) (Pakdel et al. 1989). As described earlier, another study in rainbow trout also supported this hypothesis, albeit on a much longer time scale, and using actual peak times for each mRNA (Pakdel et al. 1991). All these data support a very tightly regulated interaction between these different genes dependent on age, gender, time, and concentration of hormone. Future studies in this field should identify how estrogen mimics can interfere with endogenous primary and delayed primary responses, and how those disruptions can manifest themselves *in vivo*, probably during reproduction and development when these responses are thought to be most sensitive.

Differential mRNA Regulation by Estradiol

Estradiol is thought to regulate many physiological actions, most of which are thought to occur through the ER. Because the ER itself is auto-regulated, this serves as an additional level of interaction between E_2-regulated genes. The primary mechanism of E_2 action is through the ER, which then binds to estrogen response elements leading to up

or down regulation of a variety of genes. Unfortunately only a handful of genes have actually been identified that are regulated by E_2-ER directly. To this end, differential mRNA display RTPCR was done to identify the fingerprint of E_2 gene regulation over time, as well as to possibly identify novel targets of E_2-regulation.

As shown in Figures 2-14 and 2-15, there are many changes in mRNA expression that occur over 48 h. Arrows with double lines on the right indicate mRNA populations that are transiently up regulated and regular arrows on the left point out mRNAs down regulated over 48 h (Figure 2-14). In Figure 2-15, regular arrows on the right indicate up regulated mRNAs and stippled arrows on the left represent mRNAs not affected by E_2-exposure over 48 h. Several of the bands of interest (indicated by arrows) were excised from the gel, cloned, and sequenced. So far only two mRNAs have been positively identified. The first was Vtg, and as described earlier this was validated extensively as estrogen inducible. The second mRNA identified, a protein disulfide isomerase-related protein termed ERp72, appears to be a novel target of estrogen transcriptional regulation.

The predicted amino acid sequence of LMB ERp72 aligns with high similarity to the human, rat, and mouse ERp72 sequences (Figure 2-16A). Because this sequence is at the 3'-end it is possible to identify the retention signal at the carboxy-terminus of the protein (-KDEL) that is diagnostic of this family of endoplasmic reticulum membrane bound proteins (Mazzarella et al. 1990). This family of proteins were initially characterized by their redox capacity in catalyzing disulfide bond formation (Freedman, 1984). There have been many other functions attributed to this family of enzymes including thioredoxin activity (binding to thyroxine) (Freedman, 1989), binding to a variety of sequence non-specific peptides (Noiva et al. 1993, Spee et al. 1999); binding to

calcium (Van et al. 1993), assembly and folding of antibodies (Iida et al. 1996) and presumably binding to the estrogen receptor itself (Landel et al. 1994).

Another function of ERp72 is assisting in the folding and lipidation of apolipoprotein B (Linnik & Herscovitz 1998). This is very interesting since Vtg belongs to the apolipoprotein gene family (Wahli et al. 1981). If ERp72 was partially responsible for folding Vtg, then it would be expected to be up regulated by E_2 to accompany the increased synthesis of Vtg. In fact, it was reported that this family of protein disulfide isomerase proteins are preferentially expressed in cells actively secreting proteins (Hayano & Kikuchi 1995). Even more interesting, but perhaps confusing is a report providing evidence that the ERp72 actually binds estradiol. In fact a portion of the ERp72 amino acid sequence actually shares two spans of high homology to the hormone binding domain of estrogen receptor (Tsibris et al. 1989).

This LMB ERp72 cDNA clone was used as a probe in Northern blot experiments to validate its estrogen regulation (Figure 2-16B). It appears that LMB ERp72 is very rapidly induced following E_2-injection. In fact it seems that it is maximally induced by 1 day post injection, and quickly returns to just above basal levels by 2 to 4 days (Figure 2-16B and 2-16D). This is intriguing given the context of what was investigated regarding ER and Vtg mRNA induction in these same samples. It would appear that ERp72 may not require the induction of ER mRNA to synthesize its peak levels like Vtg mRNA is thought to. If ERp72 serves some function in Vtg protein folding, then it makes sense that it is up regulated prior to Vtg mRNA synthesis. Very limited information is known on how ERp72 is regulated (Dorner et al. 1990). There is some evidence that another protein disulfide isomerase family member, ERp61 (HIP-70), is under hormonal control (Kaplitt et al, 1993). Since ERp72 has not been previously documented as estrogen-

inducible or even identified in teleosts, much more characterization needs to be done to better understand its transcriptional regulation and function in the overall estrogen response. In addition to ERp72, the other E_2-regulated mRNAs illustrated in Figures 2-14 and 2-15 need to be identified and characterized, perhaps leading to a better understanding of the family of responses to estradiol.

Table 2-1. Plasma Vtg levels 48 hours post E_2 injection

dose*	E_2	EE_2	NP	MXC	DDT
0.00	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)
0.0005	0.004 (0.009)	N/A	N/A	N/A	N/A
0.0050	0.018 (0.025)	4.358 (1.602)	N/A	N/A	N/A
0.0500	0.480 (0.402)	7.978 (1.743)	0.005 (0.005)	0.000 (0.000)	0.007 (0.010)
0.5000	3.310 (1.471)	11.783 (2.043)	0.002 (0.006)	0.003 (0.008)	0.007 (0.022)
5.0000	8.846 (1.035)	N/A	0.000 (0.000)	0.000 (0.000)	0.003 (0.006)

*Dose is mg chemical/Kg body weight (ppm)
Data are presented as the mean mg vitellogenin/mL plasma (standard deviation)
N/A is not applicable

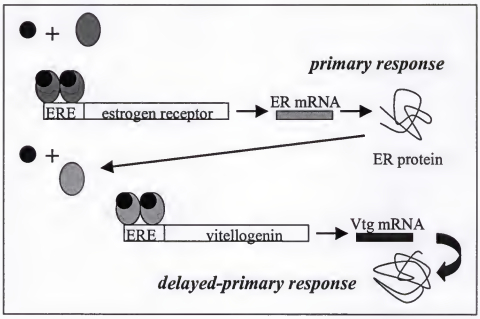

Figure 2-1. Largemouth bass mRNA coordination hypothesis. Following acute estrogen stimulation, ER and Vtg mRNA would be represent primary and delay primary responses, respectively, over time.

Figure 2-2. Largemouth bass (*Micropterus salmoides*).

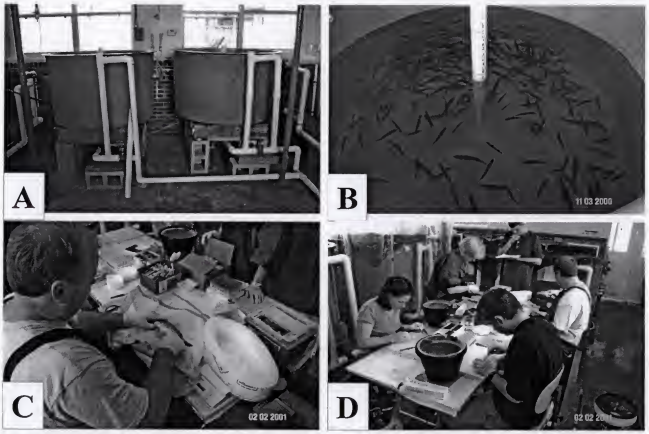

Figure 2-3. Largemouth bass sampling by lab personnel. A) 150 gallon tanks used for experiment; B) Largemouth bass in a tank; C) Kevin Kroll collecting first sample; D) Assembly line used to process samples on site.

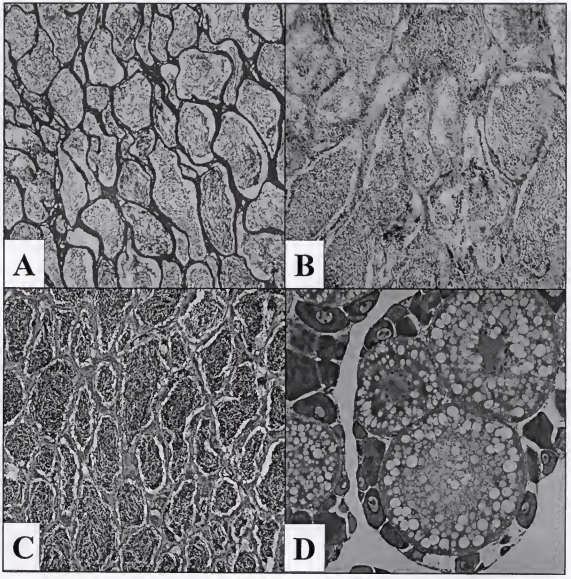

Figure 2-4. Histology of largemouth bass gonads. A) Male LMB sampled in July with low to no observable spermatogenic activity; B) Male LMB sampled in November with low to moderate spermatogenic activity; C) Male LMB sampled in January with moderate to high spermatogenic activity; D) Female pre-vitellogenic oocytes.

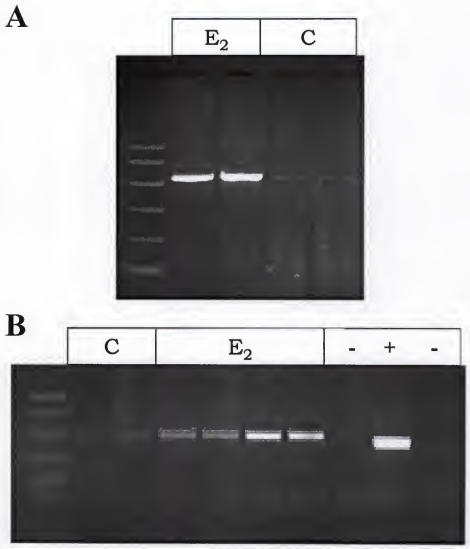

Figure 2-5. Estrogen receptor and vitellogenin RT-PCR products. RNA is from *in vivo* E_2-exposed largemouth bass samples, all of the expected size. A) RT-PCR using ER specific primers on E_2 and control RNA; B) RT-PCR with Vtg primers on control, 24 and 48 h post E_2-injection. Size markers on the left of both gels are as follows from top to bottom: 1000, 750, 500, 300, and 150 bp.

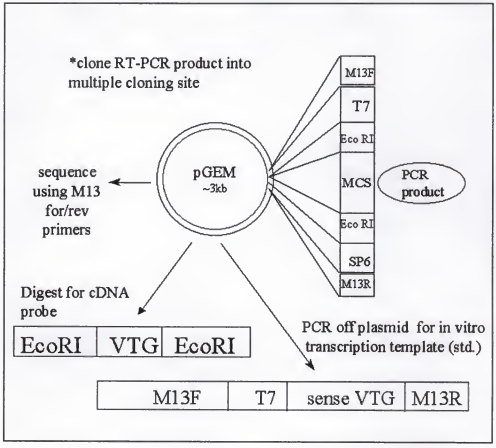

Figure 2-6. Cloning strategy for estrogen receptor and vitellogenin PCR products. The system was used to generate templates for cDNA probes, cycle sequencing with M13 primers, and *in vitro* transcription of cRNA standards.

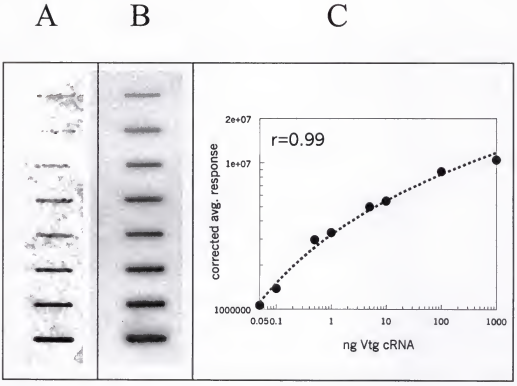

Figure 2-7. Largemouth bass vitellogenin cRNA standard curve. Performed using *in vitro* transcription; A) Methylene blue stain of *in vitro* synthesized cRNA standards (serially diluted) prior to hybridization; B) Standards probed with VTG cDNA probe (following hybridization); C) Data recovered from phosphorimager plotted against the known cRNA concentrations to create a standard curve for that specific blot, r= 0.99.

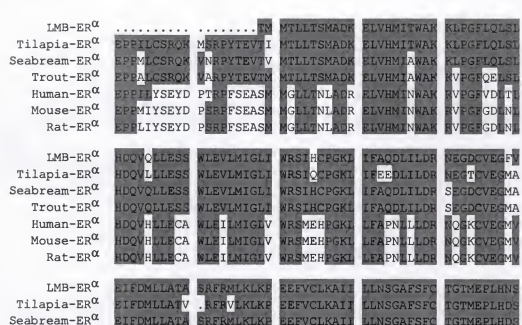

Figure 2-8. Multiple sequence alignment of cloned largemouth bass estrogen receptor fragment. Predicted amino acid sequence for largemouth bass ER from PCR amplified sequence.

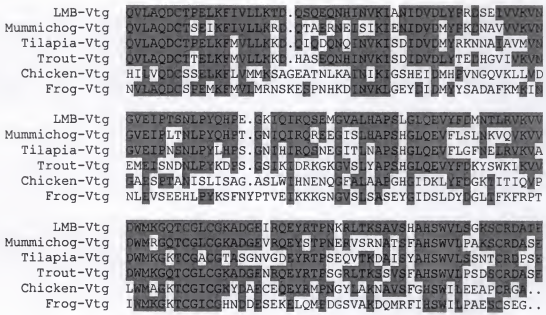

Figure 2-9. Multiple sequence alignment of cloned largemouth bass vitellogenin fragment. Predicted amino acid sequence for largemouth bass Vtg from PCR amplified sequence.

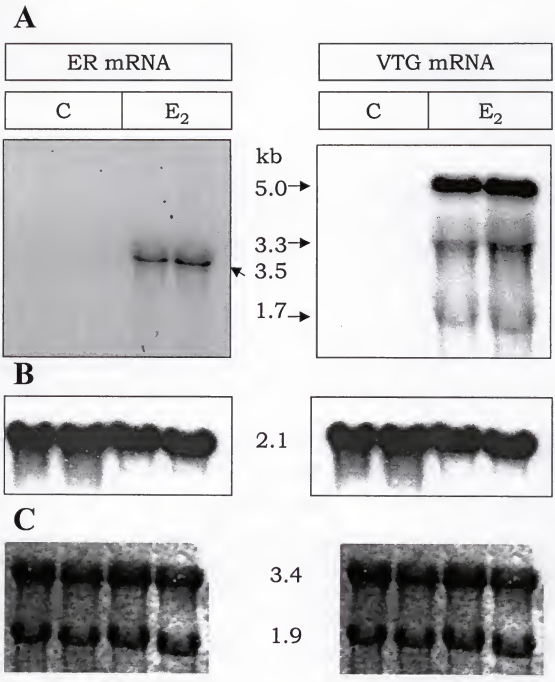

Figure 2-10. Largemouth bass liver mRNA characterization. Northern blots of control and exposed livers in duplicate. A) Estrogen receptor and vitellogenin mRNAs; B) β-actin mRNA; C) rRNAs stained with methylene blue.

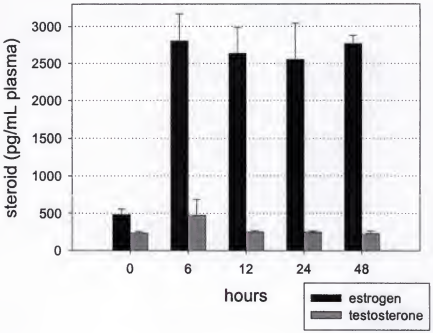

Figure 2-11. Plasma steroid measurements out to 48 h post injection. These fish were adult LMB injected once with 2 mg/Kg E_2. Male LMB sampled in July with low to no observable spermatogenic activity.

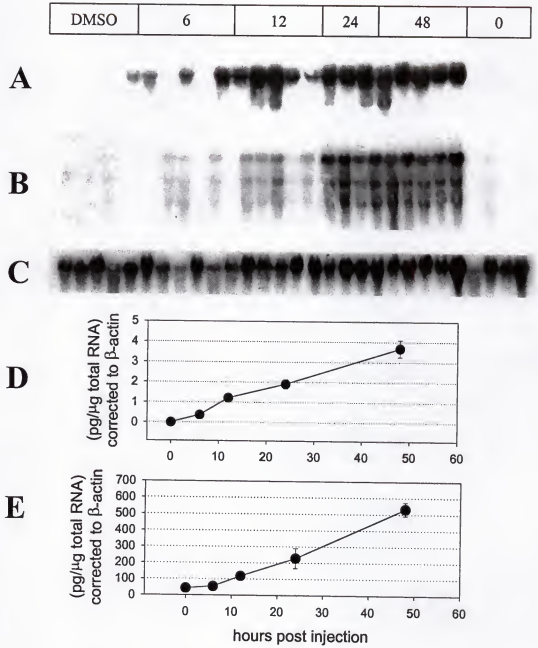

Figure 2-12. Estradiol induced mRNAs over 48 hours. A) ERα mRNA mRNA by Northern; B) Vtg mRNA by Northern; C) β-actin mRNA by Northern; D) ERα mRNA corrected to β-actin by slot blot; E) Vtg mRNA corrected to β-actin by slot blot.

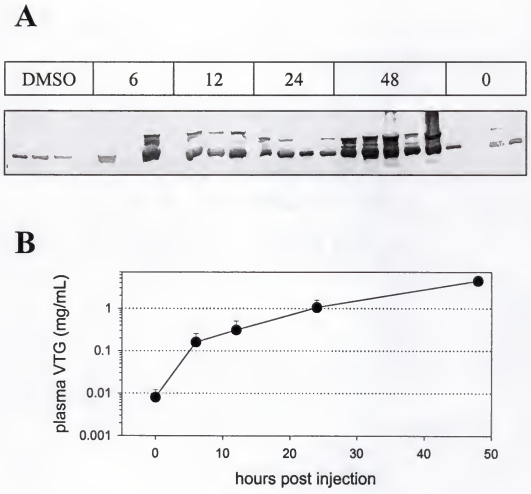

Figure 2-13. Plasma vitellogenin induction over 48 hours. A) Western blot demonstrating the two Vtg monomers (180 and 160 kDa) induced by estradiol; B) Quantitation of Vtg induction by ELISA using the same set of samples.

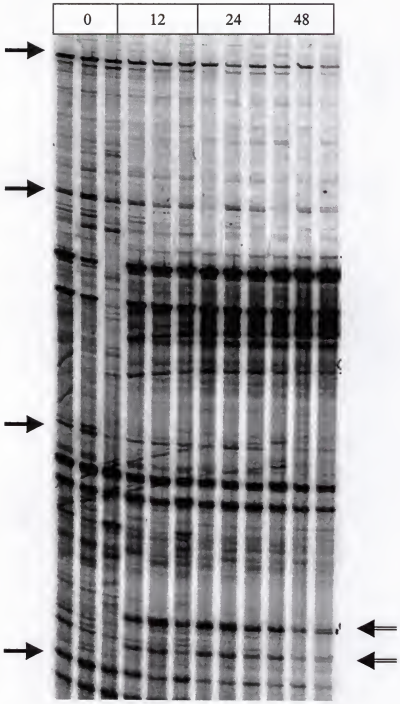

Figure 2-14. Largemouth bass differential display primer pair G-23. Arrows on left represent mRNAs down regulated over time following E_2 treatment. Arrows on right represent transient increases in specific mRNAs. Three separate fish liver RNA (lanes) analyzed per timepoint. Samples were taken over 48 h.

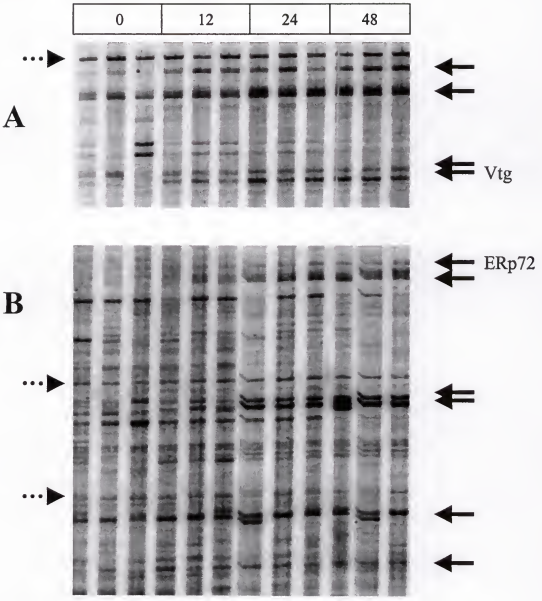

Figure 2-15. Largemouth bass differential display using primer pairs G-10 and C-1 . Arrows on left represent mRNAs constitutive over time following E_2 treatment. Arrows on right represent up regulation of specific mRNAs. Vtg and PDI are two of the identified mRNAs up regulated by E_2. Three separate fish liver RNA (lanes) analyzed per time point. A) Primer pair G-10; B) Primer pair C-1. Samples taken over 48 h.

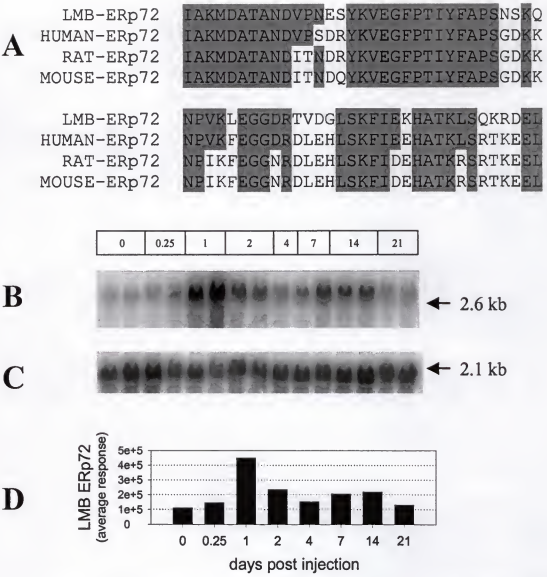

Figure 2-16. Verification of largemouth bass ERP72. A) Multiple sequence alignment of predicted amino acid sequence lined up against some mammalian species; B) E_2-induction of LMB ERp72 mRNA over 21 days by Northern analysis; C) β-actin mRNA on same Northern blot; D) Relative quantitation of ERp72 mRNA induction by E_2.

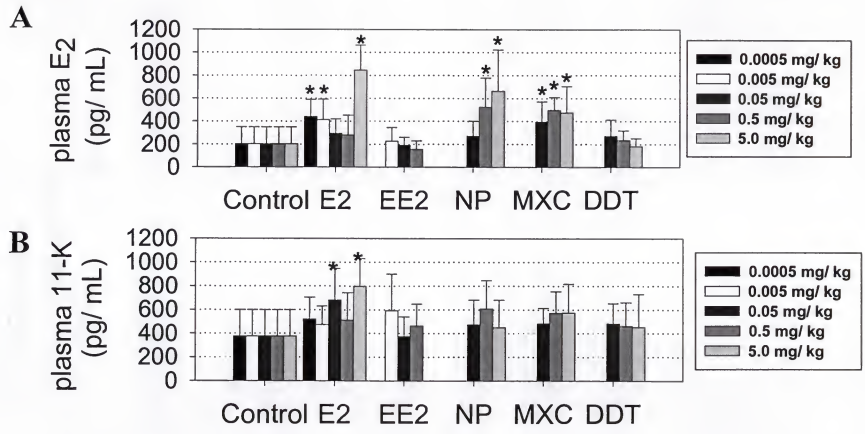

Figure 2-17. Plasma steroids 48 hours following exposure. All data is from male adult LMB. A) Plasma estradiol; B) Plasma 11-ketotestosterone (11-KT). Astericks indicate experimental groups that were significantly different from controls ($P<0.05$) using the Student's t-test.

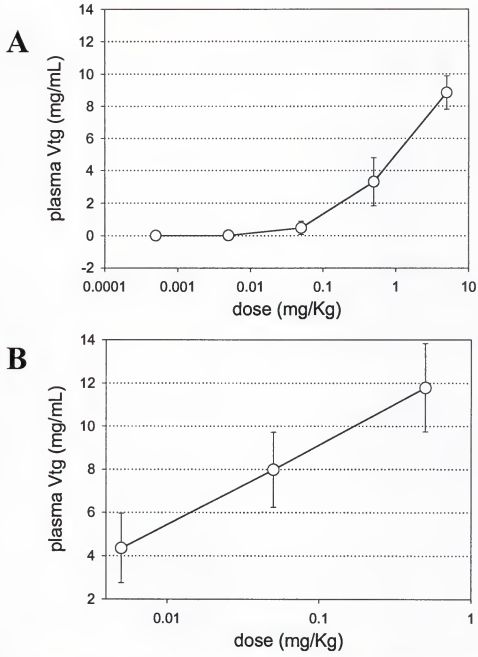

Figure 2-18. Dose response induction of plasma vitellogenin in largemouth bass. A) Estradiol-induced plasma vitellogenin at 48 h post injection; B) Ethinylestradiol-induced plasma vitellogenin.

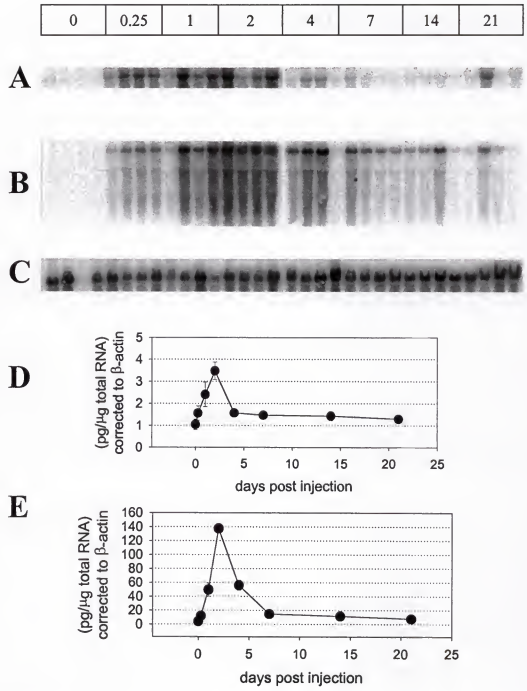

Figure 2-19. Time course of estradiol induced mRNAs over 21 days. A) ERα mRNA mRNA by Northern; B) Vtg mRNA by Northern; C) β-actin mRNA by Northern; D) ER α mRNA corrected to β-actin by slot blot; E) Vtg mRNA corrected to β-actin by slot blot.

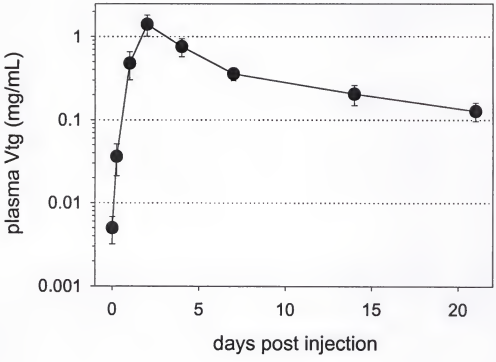

Figure 2-20. Time course of plasma vitellogenin induction. Quantitation of vitellogenin by ELISA.

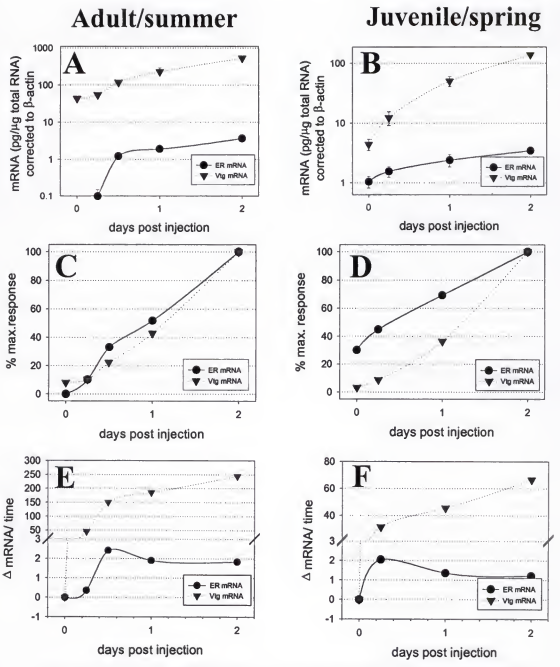

Figure 2-21. Largemouth bass estrogen receptor and vitellogenin mRNA comparison. The induction of ER and Vtg mRNA over 2 days are illustrated together three different ways. The data on the left is from adult male LMB taken during summer. The data on the right is from juvenile male LMB taken during spring. A) and B) raw mRNA data; C) and D) mRNA data plotted as a percent of the maximal response; E) and F) mRNA data plotted as the increase in mRNA divided by time.

CHAPTER 3
DEVELOPMENT OF AN *IN VITRO* MODEL: LARGEMOUTH BASS PRIMARY HEPATOCYTES

Introduction

The use of fish primary hepatocytes as a model system represents a relatively new approach to characterizing the cell biology and molecular physiology of teleosts (Baski & Frazier 1990, Hightower & Renfro 1988, Moon et al. 1985). Part of understanding gene conservation and evolution involves cross species biology. The significance in understanding an environmentally relevant species (such as largemouth bass) on a molecular level will help explain how xenobiotics may be impacting our environment. The existing approaches to this problem include field studies, very limited *in vivo* controlled exposures, and more recently, recombinant yeast expression assays. The field studies are almost entirely observational and do not provide detailed insight into possible mechanisms of xenobiotic disruption. Controlled *in vivo* exposures are limited because of the availability of healthy tank-raised fish. Recombinant yeast assays continue to be of limited value because of incomplete representation of tissue metabolism and intracellular protein constituents, both present in vertebrates and critical to target gene regulation.

Fish primary hepatocytes were initially developed and characterized using goldfish and rainbow trout (Baski & Frazier 1990). More recently, the demand for new models has led to cultures being isolated from various other aquatic organisms such as eel, catfish, and flounder. In a more general sense, largemouth bass may serve as a sentinel species in field population data because of its predatory nature and apparent

environmental sensitivity. However, studies using largemouth bass (LMB) as a model are very scarce in the literature, despite its importance in sports and recreation in the southeastern United States. Following development of this assay, it is anticipated that xenobiotic studies can be conducted using LMB and will constitute the first report on the characterization of this fish at the molecular level.

Rationale

One of the primary mechanisms of action for hormones is to bind to cognate intracellular receptors and induce *de novo* transcription. Based on this, most of the *in vitro* assays used to assess estrogenicity (the most studied mechanism of endocrine disruption) rely on this ligand-receptor mediated response. These methods include receptor binding, cell proliferation, receptor-dependent gene expression, and recombinant receptor-reporter gene constructs (Jensen & Jacobson 1960, Jobling & Sumpter 1993, Soto et al. 1995, White et al. 1994). Each of these assays has distinct advantages and disadvantages (Zacharewski, 1997, Zacharewski, 1998). All these assays are useful in determining very specific aspects of the predicted mechanism of action (receptor binding), even in so far as to allow receptor-DNA interaction followed by transcription initiation (gene expression and gene constructs). They are easily adapted to large scale screening and do not require the use of animals. Cell proliferation is a simple functional assay of estrogenicity, unfortunately there are few estrogen-responsive cell lines. While the in vitro assays are useful, they each have disadvantages as well. Receptor binding and a number of reporter gene constructs cannot distinguish between agonists and antagonists. Xenobiotics that require metabolic bioactivation (proestrogens) will not be detected by a majority of these assays, with the exception of assays using primary

hepatocytes. Most of the yeast or cell line assays cannot account for *in vivo* pharmacokinetics.

The ability of *in vitro* assays to accurately predict *in vivo* responses of hormone mimics is limited. Disruption of the endocrine system can include multiple pathways involving different organs with unique chemical sensitivities at critical windows in time. Because of the complexity in potential pathways, it is difficult to isolate tissue-specific mechanisms of action. For xenoestrogens in particular there also seems to be non-receptor-mediated effects on the cell. As discussed earlier, ligand-independent receptor activation can also elicit responses. The *in vivo* mechanisms of specific gene regulation and expression depend on the presence and state of intracellular constituents, which are not always accurately represented by transfected reporter assays in transformed cell lines or yeast. In fact, using yeast or cell lines, one must transfect and express estrogen receptors since these systems lack these most basic elements in the pathway. This begs the question of what other regulatory cofactors are missing in these systems? Therefore, the importance of an unaltered vertebrate system with endogenous markers is critical to understanding how the mechanism of xenoestrogen disruption may interfere with the reproductive and developmental success of an individual. As discussed below, primary fish hepatocytes may fulfill this need *in vitro* without additional modifications and including endogenous markers necessary to investigate specific mechanisms of xenoestrogen action.

Fish Primary Hepatocytes

To better understand how contaminants are impacting the biological systems, very specific molecular responses need to be quantified using an unaltered cellular system that

accurately represents *in vivo* end points. Fish primary hepatocytes, which retain an active estrogen receptor and intact metabolic pathways can provide this at the cellular level (Baski & Frazier 1990, Flouriot et al. 1993, Hightower & Renfro 1988, Moon et al. 1985). This system can also be used to measure endogenously expressed markers, such as vitellogenin and estrogen receptor mRNAs that can be directly correlated with *in vivo* experiments (Anderson et al. 1996).

To best study the mechanisms of disruption of specific xenoestrogens, an ideal model would share the *in vivo* effects of bioactivation and metabolism with the feasibility for extensive experimental manipulation provided by an *in vitro* system. Because primary hepatoctyes still retain many of the functions of liver tissue, they respond similarly. Among the functions they retain is the ability to metabolize compounds, bind hormone active agents through the endogenous estrogen receptors, and recruit intracellular proteins to initiate *de novo* transcription (Baski & Frazier 1990). This is particularly important for contaminants such as methoxychlor that have been shown to confound mammalian cell transactivation assays, as it requires metabolic activation (Bulger et al. 1978, Shelby et al. 1996). Therefore the capacity of primary hepatocyte cultures to retain more of the original organ functions than immortalized or transformed cell lines will allow for more relevant mechanistic studies of hormone-regulated gene expression and metabolism. The reason is that these cell lines often lose normal cellular controls on gene regulation. Fish hepatocytes are also valuable for their importance in steroid metabolism, as well as being the site of vitellogenin biosynthesis (Baski & Frazier 1990). This model has proven itself to be valuable in understanding various effects of teleost liver metabolism including hormonal regulation, and protein

metabolism/expression (Moon et al. 1985). This is especially important for these studies since fish primary hepatocytes have been shown to be estrogen-responsive and still express an active ER, whereas cell lines do not generally share these characteristics (Pelissero et al. 1993).

For various reasons, primary fish hepatocytes better represent *in vivo* cellular conditions in fish than mammalian hepatocytes do for mammalian systems. The structure of fish livers is not organized into a hepatic triad, rather it is remarkably homogenous. Fish livers are also roughly 80% hepatocytes, i.e., predominantly one cell type; whereas mammalian livers consist of only 60% hepatocytes with other cell types making up the other 40% of the population (Blair et al. 1990, Hampton et al. 1985). Therefore fish primary hepatocytes can potentially represent *in vivo* cellular and intercellular actions very well.

Objective

The majority of information on fish primary hepatocytes has been collected using the rainbow trout as a model. There seems to be as many different ways to isolate and culture this cell type as there are publications on the method. Many different techniques of isolation and culturing of hepatocytes were attempted with largemouth bass. The first technique was using an adaptation of Ostrander and Blair from trout (Blair et al. 1990, Ostrander et al. 1995). Because LMB hepatocytes did not thrive under those conditions, different perfusion buffers and incubation media were used. Non-perfusion methods of cell dissociation relying on mechanical and chemical techniques (Freshney, 2000) were also tried. Ultimately the most promising technique was using a method developed by Mommsen (Mommsen et al. 1994). The objective was to develop and validate a primary

hepatocyte model that could be used to examine the differential molecular mechanisms behind gene regulation by various xenoestrogens.

Materials and Methods

Fish Collection and Maintenance

Largemouth bass (*Micropterus salmoides*) were purchased from American Sportfish Hatchery (Montgomery, Alabama). They were either maintained at the Aquatic Toxicology Facility Lab at the University of Florida under the direction of Dr. Evan Gallagher or at the United States Geological Survey- Carribean Science Center under the direction of Dr. Timothy Gross. Fish were held in aerated 104 to 250 gallon fiberglass tanks, under constant conditions of 21 +/-2° C prior to hepatocyte isolation. Dissolved oxygen, total ammonia content, and pH were monitored during this time. The fish were exposed to ambient light concentrations, and fed Aquamax 5D05 fish feed (Purina).

Reagents and Equipment

If not specifically indicated, all reagents were purchased from Sigma-Aldrich or Life Technologies. For hepatocyte isolation, specialized surgical tools from Roboz Surgical (Rockville, MD) and Fisher Scientific were used. For perfusing the liver, IV-cannulae, IV extension tubing, and silk sutures (3.0) were obtained from the nearby hospital supply center. Collagenase Type I and IV were utilized from Sigma and Worthington Biochemical.

For primary hepatocyte culturing, 6, 12, or 24-well Becton-Dickenson-Falcon Primaria (Bedford, MA) tissue culture plates were used. Charcoal-stripped fetal bovine

serum was obtained from HyClone laboratories (Logan, UT). Clinical kits to determine lactate dehydrogenase activity were used from Sigma and Promega.

RNA isolation and cDNA amplification were performed as described in Chapter 2 of this dissertation and previously (Bowman & Denslow 1999). Briefly, the individual tissue culture plate wells were prepared for RNA using the acid phenol guanidinium-isothiocyanate method (Chomczynski & Sacchi 1987) or using RNeasy columns from Qiagen following the manufacturer's instructions. Total RNA isolates, resuspended in water, were measured at 260 and 280 nm using a spectrophotometer. The 260 nm reading was used to estimate the concentration of total RNA recovered from the isolation. The 260/280 ratio as well as a 1% agarose-formaldehyde gel stained with ethidium bromide were used to verify the quality of the RNA in each sample. Oligo-dT primers, dNTPs, 5X transcription buffer (Life Technologies), and Superscript II (Life Technologies) were used to reverse transcribe 2 μg total RNA. Vitellogenin (Vtg) primers (80 pmol/μL) or beta actin primers (10 pmol/μL) in10X reaction buffer with $MgCl_2$ (Perkin-Elmer), dNTPs, AmpliTaq (Perkin-Elmer,), and 2 μl of cDNA from the reverse transcription reaction were used to amplify portions of these respective genes by the polymerase chain reaction (PCR). The conditions for the PCR were: hold at 80°C for 3 min; hold at 94°C for 3 min; 35 cycles of 94°C for 45 sec, 52°C for 90 sec, 72°C for 45 sec; hold at 72°C for 10 min; and hold at 4°C for one hour. The PCR products were analyzed and purified by 1.2% agarose gel electrophoresis. These RT-PCR reactions using vitellogenin or beta actin primers were performed as described in Chapter 2 of this dissertation and in the literature (Bowman & Denslow 1999).

For both isolation and culturing of primary cells there was a necessity of several pieces of equipment. For the perfusion of the organ, a peristaltic pump with manual speed controls was necessary to adjust flow. For washing of isolated cells, a swinging bucket centrifuge was necessary. Final culture and maintenance of hepatocytes required a laminar flow biological hood, water-jacket cooled incubator, and external chiller to maintain temperatures below ambient in the incubator. For characterization, viability, and counting, an inverted and regular light microscope outfitted with Pixera Viewfinder Pro (Los Gatos, CA) and analyzed using Pixera Studio Pro (Los Gatos, CA) were used.

Transmission electron microscopy was performed using a Hitachi H-7000 at 75 kV in the Electron Microscopy Core at the University of Florida. First, isolated hepatocytes were fixed by 2% glutaraldehyde in PBS. After washing with PBS, the cells were post-fixed in 1% OsO_4 for one hour. The cells were then washed again in PBS and water, and then dehydrated with graded ethanol, followed by acetone. The sample was then infiltrated and polymerized by graded epoxy resin (Embed 812), followed by sectioning with an ultra microtome. The section was then shaped using a diamond knife to ~70nm/slice. Once on a mesh, the sample was stained with uranyl acetate and lead citrate prior to examination by the electron microscope. Sample preparation and digital image capturing was done in collaboration with individuals in the Electron Microscopy Core.

Buffers and Media

There are three buffered solutions necessary for hepatocyte perfusion and isolation. All are modifications of Hanks balanced salt solutions (Hanks and Wallace, 1949). The first solution serves as a pre-perfusion buffer to remove all the blood from

the liver prior to enzymatic digestion. The second solution contains 105 to 131 U/mL collagenase type I or IV. This bacterial collagenase (EC 3.4.24.3) is from *Clostridium histolyticum*. This is used during perfusion to dissociate cell to cell interactions within the liver. The third solution is used to wash the initial isolated cell pellet. It contains calcium and bovine serum albumin to maintain cellular integrity during washing. All buffers require sterile 0.2 micron filtration prior to use. See Table 1 for individual components of the three buffered solutions.

After experimenting with other prepared media such as Dulbucco's Minimal Essential Medium + Ham's F-12, largemouth bass hepatocytes seemed to respond best to a modified Leibovitz's (L-15) media. Important are the bicarbonate and HEPES buffering components to maintain necessary physiological pH. In addition, fungizone, streptomycin, and penicillin were added to prevent contamination. Media requires sterile 0.2 micron filtration prior to use. Typically 3 to 5% charcoal-stripped fetal bovine serum was used, but the best amount and the impact of serum was not fully investigated. See Table 3-2 for individual components of the medium used for culturing largemouth bass primary hepatocytes.

Hepatocyte Isolation

Two essential steps in a largemouth bass primary hepatocyte culture experiment are the isolation of a homogenous population of hepatocytes and the culturing of these isolated cell types. The latter step will be discussed in the next section. The first step is dissociating all cell types in the liver, then selectively removing viable hepatocytes. The other cell types include biliary epithelial cells, perisinusoidal fat-storing cells of Ito,

melano-macrophages, sinusoidal endothelial cells, and red blood cells. This first isolation step is very technically challenging and is dependent on a healthy fish liver and successful cannulation of the portal vein.

The Fish

Largemouth bass may be distributed nationally and live in most bodies of freshwater, but they have yet to be successfully reared in tanks. In fact, this fish species has to be raised on artificial feed from birth in order to control the diet. Largemouth bass raised on artificial feed are ideal since diet may have an impact on the interpretation of experimental results. For primary fish hepatocytes it is important to have a consistent and reliable source of fish. For long-term maintenance, LMB should be kept in small ponds and fed artificial feed. A small population can be kept in tanks for easy access, but only for short periods of time, since it has not yet been demonstrated that LMB can survive long-term in tanks. For purposes of this research, juvenile or male fish were used for experiments in estrogen-regulated gene expression to minimize host female-specific steroid influences. Obviously larger fish have larger livers and that usually results in a larger yield of hepatocytes per experiment. Even though small LMB (100 g) have smaller vessels, they are suitable to work with, and should provide successful hepatocyte isolation.

The Perfusion

Primary hepatocyte isolation involves two-step perfusion of the liver by cannulating the hepatic portal vein with retrograde flow out of the heart (Figure 3-1). The objective of the particular technique is to utilize the blood vessel distribution of the liver to administer the enzyme collagenase. This enzyme delivered in solution will

dissociate cell-to-cell interactions of the organ, resulting in a cell suspension within the organ "pouch". Once the cells are dissociated, hepatocytes can be differentially selected and recovered in culture.

Following proper anesthetic (150 ppm MS-222), the fish is injected intravenously with heparin (10,000 U/Kg) to prevent blood clotting during the procedure (LMB have a very active clotting mechanism). The fish is opened with a long ventral incision from anus up to the mouth. Following lateral incisions on both sides, the ventral skin is removed or pinned back to expose the viscera. Because of the importance of sex determination, the gonads are removed for quick microscopic verification. Connective tissue surrounding the liver is removed, exposing the gall bladder and hepatic portal vein. First the bile is removed from the gall bladder using a 1cc tuberculin syringe in order to prevent contamination of liver cells. Then two loose square knots are tied around the hepatic portal vein with silk sutures (3-0). Holding the hepatic portal vein with tweezers a couple of centimeters posterior to organ entry, an IV-cannulae is inserted carefully into the vessel. Once in, the cannulae is slowly advanced toward the organ while removing the needle. Just prior to organ entry, the cannulae is secured in place using the two prelooped silk sutures. Then the cannulae is attached to IV extension tubing which is attached to a flask of solution 1 containing oxygen via a peristaltic pump (Figure 3-1). Once all the tubing is connected, the pump is started very slowly, and solution movement is monitored very closely. If there is no leakage by the cannulae and the organ bloats upon solution entry, then the heart is quickly clipped to allow for release of pressure. Solution 1 is pumped slowly (2 mL/min) through the organ in situ for 5 to 10 min in order to remove all traces of blood from the organ. The liver should blanch in color as

the blood is pushed out. The liver is gently massaged with a glass rod during perfusion. After perfusion with solution 1, the perfusion buffer is switched to solution 2 containing collagenase. Once solution 2 reaches the organ, the collagenase will disrupt cell-to-cell interactions (flow ~2 mL/min). The liver should lighten in color as blood is removed and collagenase has time to digest. Gentle massage of the organ will facilitate this dissociation. After 15 to 25 min, the organ will feel soft and mushy. The liver is then removed from the fish, with care being taken to remove all connective tissue without nicking the "bag of cells".

The Isolation of Hepatocytes

Following perfusion, it is necessary to isolate hepatocytes from the other cell types in the liver. The liver is minced with sterile scalpels in a petri dish containing solution 3. The cell suspension is gently filtered through coarse (~254 um) and fine (~73 um) screens into a centrifuge tube. Very diagnostic at this stage is the retained material on the screens (Figure 3-2A). If it is slimy or oozes rather than drips, then the cells are likely overdigested (too much collagenase or too long). If cells clump when put in solution 3, then the liver was underdigested. To differentially select for hepatocytes, the cell suspension is centrifuged at 50xg for 3 min. The dilute supernatant will contain lighter cell types such as red blood cells (Figure 3-2B). At this light centrifugation, only hepatocytes will pellet since they are the heaviest and largest cell type present (Figure 3-2C). This pellet, predominantly hepatocytes, is washed in solution 3 several more times. In the last wash, the pellet is resuspended in media and allowed to settle for 60 min on ice. This last settling at 1 x g increases the homogeneity of hepatocyte preparation. After pouring off the supernatant, the now goopy pellet is resuspended and washed once more

in media. This last pellet (Figure 3-2D) is resuspended in 5 to 20 mL media containing serum and is ready for culture.

Using transmission electron microscopy, cell populations were visualized following isolation. This technique positively identified that the population recovered was >99% hepatocytes as defined by their distinctive morphology. These cells had extensive rough endoplasmic reticulum, visible golgi, characteristic nuclear chromatin arrangement, glycogen deposits, and mitochondria aplenty (Figure 3-3). The only other cell types that were found were an occasional red blood cell, and another cell type which is likely a perisinusoidal fat-storing cell of Ito, as characterized by the cell shape, fibrous matrix, lipid droplets, and phagosomes (Figure 3-3).

Hepatocyte Culture

This step in the fish primary culture experiment should be more consistent with those techniques described for mammalian cells, but due to some known and unknown unique features, this does not hold true. The most striking difference perhaps is their incubation temperature, at or below ambient. Whereas most mammalian cells prefer incubation around 37°C, most fish cultures, such as rainbow trout, operate around 12 to 16°C since that is their native temperature. One of the unique features of working with largemouth bass is that they are native to more temperate latitudes and therefore the cultures were grown at or below 25°C.

Cell Viability

Once isolated cells are resuspended in media, their approximate numbers are determined using a hemacytometer. In order to accurately quantify the number of viable

cells, a diluted cell subsample is mixed with trypan blue. Viable cells will exclude the dye, while dead cells will look blue because they cannot repel it (Figure 3-4). This cell suspension is put on a hemacytometer, allowing for the "yellow" cells to be counted. These cells are large and have a glow to them, unlike cytoplasmic blebs that are smaller and a flatter shade of yellow. A typical viable cell yield is between 0.5 and 7 x 10^7 cells. This measure of cell viability was only obtained prior to plating of cells, since once attached it was difficult to transfer the cells.

Lactate dehydrogenase (LDH) activity was another measure of cell viability that was evaluated for these cells. This enzyme activity was tested in the media relative to that in the cell layer. The idea is that LDH is a liver specific enzyme that should not be present in the media, unless the cell membrane is compromised and LDH is leaked out of the cell. Therefore more LDH in the media relative to the cell over time indicated compromised cell viability. The assay is simple and can be measured using different kits available from various manufacturers. An example comparing two different kits, Sigma and Promega, illustrates the variability in this viability assay (Figure 3-5).

Because it was difficult getting this culture system to induce expression of the target gene, vitellogenin, culture conditions were constantly being modified. The ultimate viability test was to isolate intact RNA and induce Vtg over time. Among other things, the cell density, well size, percent serum, type of serum, plate substrate, temperature, and other incubation conditions were constantly being optimized. These changes limited the capacity to fully characterize the viability of the culture over time. The media was also regularly monitored for pH fluctuations. Osmolality of the media

and perfusion buffers were measured and found to be similar to that of LMB plasma, potentially ruling out these as viability factors (Figure 3-6).

Culture Morphology

Once approximate concentration of viable cells was determined, 0.5 to 1.0 x 10^6 cells per 10 mm diameter surface area (well) gave the most consistent results. More or less than this was not ideal cell density. While cells at higher concentrations could survive, they exceeded a monolayer and would pile up. Much less would not yield sufficient RNA. When these cells were put on Primaria plates from Becton Dickenson, they attached and spread out consistently. Over time the individual cells seemed to form cell clusters. This phenomena was best documented using an inverted light microscope and watching them over time (Figure 3-7). Healthy cells were very large, round, attached strongly, and seemed to glow. Using other treated or untreated tissue culture plastic there was inconsistent attachment and monolayer formation. The type and percent of serum supplement also seemed to influence how well the cells attached or aggregated to each other. For example, 1 to 5% charcoal-stripped fetal bovine serum consistently supported viable attached cells, but a higher concentration would disrupt the osmolality of the media and compromise viability. Serum from largemouth bass (5 to 10%) was attempted (5 to 10%), but the cells would aggregate into huge piles and viability would decrease tremendously.

Experimental Results

The motivation for developing an *in vitro* system for this model was to better understand estrogen-regulated gene expression at the molecular level in an environmentally-relevant species. To this end, isolation of RNA and monitoring gene

expression was used to gauge successful isolation and culturing of LMB primary hepatocytes. Routine culture experiments were conducted exposing cells to 0.1 and 1 μM estradiol in DMSO. For example, the data shown in Figure 3-8 were from 1 μM exposures to E_2. Control cells were both untreated and treated with DMSO only. Exposures were conducted immediately following isolation, and 24, or 48 h after isolation. Cells were exposed to 1 μM estradiol for up to 72 h, with media change or addition every 24 h.

At the end of exposures, media was withdrawn from the wells, and the monolayer of cells was bathed in cell disruption and homogenization buffer (guanidinium isothiocyanate or Qiagen's RLT buffer). After 5 to 10 min cells were scraped from individual wells into microcentrifuge tubes and further steps taken to isolate RNA as described earlier. Intact RNA was obtained from all cultures that were healthy isolates from successful perfusions (Figure 3-8A). The mRNA population seemed intact, as β-actin mRNA was routinely detected at expected levels in control and exposed samples (Figure 3-8C). The peculiar result was that the estrogen responsive gene, vitellogenin, was rarely detected. In only one experiment did it seem to be up regulated by estradiol exposure at all (Figure 3-8B). In this case the cells taken from a male LMB were cultured for 24 h, then exposed to 1 μM E_2 for 24, 48, or 72 h, with control samples (DMSO only) taken at 24 and 48 h. As shown in Figure 3-8B and 3-8D, cells exposed to E_2 up regulated Vtg mRNA compared to controls. This experiment has not been repeated, but has provided evidence that the gene was present and inducible. In other instances, if at all, Vtg mRNA appeared early in the culture (female fish liver only) and deteriorated with time.

Discussion

Primary hepatocyte isolation and culture has continued to evolve from the first landmark paper using rat liver in 1969 (Berry & Friend 1969). Methods to isolate hepatocytes from teleostean fishes have been undergoing constant change for the past twenty years. Even within the most well-studied model, rainbow trout, there are countless variations on which perfusion buffers, media, serum supplements, substrate, and even dissociation enzymes provide the most prolific and consistent results (Blair et al. 1990, Flouriot et al. 1993, Klaunig et al. 1985, Kocal et al. 1988, Lipsky et al. 1986, Mommsen et al. 1994). Largemouth bass had not been used before as an *in vitro* culture model, so various methods were tried in an attempt to isolate and culture viable hepatocytes. Because of the technical complexity and time commitment, this had been a difficult model to perfect.

Initially, an elaborate gravity driven perfusion-recirculation system was used to perfuse livers ex-vivo. Due to difficulties of transferring the organ to the apparatus while cannulated, this method did not prove successful. Cannulation of the hepatic portal vein was technically difficult and keeping it in place with sutures was equally challenging. These complications motivated an altogether alternative technique not requiring perfusion of the organ. This non-perfusion technique optimized for embryos involved gentle mechanical dissociation on a stir plate following mincing of fresh tissue. This also involved chemical digestions using various hydrolytic enzymes such as trypsin, collagenase, hyaluronidase, dispase, DNase, and others. The advantages of this method was consistent technique and tailored enzyme digestion depending on downstream

experiments (some enzymes such as collagenase have protease activities that may not be desirable). The downfall of using a non-perfusion technique was a drastically low yield of viable cells, and a preparation that was not as consistent as would be expected. Ultimately, after much practice the cannulation of the hepatic portal vein was mastered using an IV-cannulae. The other change that led to successful isolation of viable cells was manually controlling the flow rate of perfusion while the organ was still in the body cavity, followed by optimization of perfusion buffers and cell isolation.

The use of general cytology stains, light microscopy, and electron microscopy were critical to determining the steps in the hepatocyte isolation procedure that worked to actually separate cell types and verify successful hepatocyte recovery. The cytology stains clearly helped evaluate the efficiency of filtering out the digested cells and helped monitor other steps including differential centrifugation (Figure 3-2). Transmission electron microscopy definitively identified that >99% of the recovered cells were hepatocytes. It also allowed us to identify what potential contaminating cell types were present, namely red blood cells. Another phagocytic cell type that was found infrequently looked like a perisinusoidal fat-storing cell of Ito (Blair et al. 1990, Ferri & Sesso 1981). All these cell types look very similar to that described and shown for fish hepatocytes in the literature (Blair et al. 1990, Brusle & Anadon 1996).

Once viable cells were isolated, a system to successfully culture the cells needed to be developed. There are three documented ways to culture fish hepatocytes: in suspension, as monolayers, or as aggregates. The best studied and easiest to maintain is a monolayer culture. There has been some suggestion that fish hepatocytes cultured as aggregates retain more representative metabolism and liver function as compared to

monolayer (Cravedi et al. 1996, Flouriot et al. 1993), supposedly because of better cell-to-cell interactions. LMB hepatocytes cultured in these different ways, but the cells attached and spread out very consistently on Primaria plates. If other tissue-culture treated plastic plates or dishes were used, an aggregate culture could be created with gentle shaking. Consistent expression and viability were better with the monolayer culture, the aggregate or suspension cultures were difficult to work with and did not survive as long as the monolayer cultures. Documentation of cellular morphology in a monolayer culture is illustrated in Figure 3-7. These cells look very similar to the morphology described and shown by multiple laboratories (Blair et al. 1990, Klaunig et al. 1985, Kocal et al. 1988, Lipsky et al. 1986).

This particular *in vitro* model is promising in understanding mechanisms of disruption for an environmentally-relevant species known to be impacted in the wild. Some troubleshooting still remains to be done before it can be used successfully. Principally, the culture conditions need to be optimized in order for the cells to be inducible by estrogens. Cell substrate may be important; some techniques suggest fish skin extracts help with primary hepatocyte function (Blair et al. 1990, Lipsky et al. 1986, Segner et al. 1994). Serum supplementation with host serum may prove beneficial (Kocal et al. 1988), although preliminary data using LMB serum would indicate otherwise. Despite using the two predominant prepared medias, L-15 and DMEM + Ham's F12, on the market for these cultures, further adjustments in buffering and salt content may be necessary. There are still countless supplements that may prove critical to these particular cells, these include but are not limited to insulin, glucagon, trace metals and amino acids, and other hormones (Segner, 1998).

In summary, largemouth bass primary hepatocytes were successfully isolated and cultured. There is still more work to be done optimizing the culture conditions to ensure estrogen-responsiveness of these cells. Estrogen receptor and vitellogenin genes are present (Flouriot et al. 1993), but their regulation does not appear to be consistent with the current culture conditions in LMB. Several avenues of experimentation remain to work out this final obstacle. Once this is overcome, this method can be used for in *vitro* screening of estrogenic compounds (Jobling & Sumpter 1993). More importantly though, we can investigate some basic biochemistry of how xenoestrogens may be disrupting gene regulation at the molecular level.

Table 3-1. Composition of buffered perfusion solutions used to isolate primary hepatocytes

	Chemical	Target concentration
Solution 1	NaCl	137.9 mM
	KCl	5.4 mM
	KH_2PO_4	0.44 mM
	Na_2HPO_4	0.33 mM
	HEPES	10.0 mM
	$NaHCO_3$	5.0 mM
	$MgSO_4$-$7H_20$	0.81 mM
Solution 2	*add the following to solution 1*	
	Collagenase	105 to 131 U/mL
Solution 3	*add the following to solution 1*	
	$CaCl_2$	1.5 mM
	BSA	2%

Table 3-2. Media and supplements used to culture primary hepatocytes

Chemical	Target concentration
L15	(Life Technologies #413000)
HEPES	10 mM
$NaHCO_3$	5 mM
Penicillin G sodium	100 U/mL
Streptomycin sulfate	100 µg/mL
Amphotericin B (fungizone)	0.25 µg/mL
Aprotinin	2 µg/mL

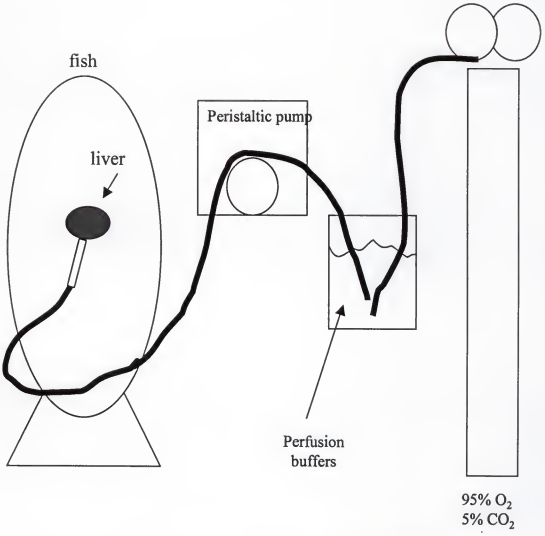

Figure 3-1. Liver perfusion set up. Largemouth bass liver cannulated and perfused with aerated buffered solutions with or without the presence of collagenase.

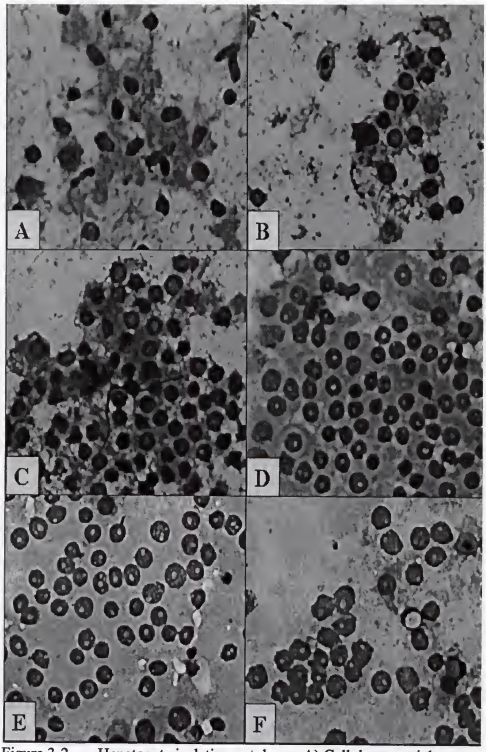

Figure 3-2. Hepatocyte isolation cytology. A) Cellular material not filtered through screen; B) Cellular material filtered through screen; C) Pelleted cells after first wash; D) Hepatocytes resuspended prior to 1 x g settling; E) Hepatocytes purified post 1 x g settling; F) Diluted hepatocytes immediately prior to culturing.

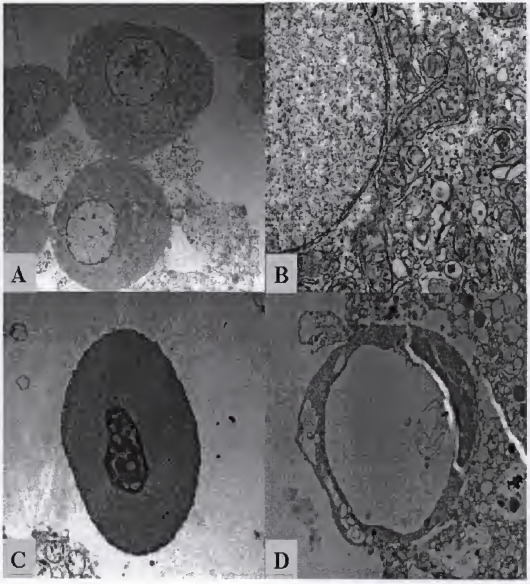

Figure 3-3. Electron microscopy of isolated hepatocytes. A) Two intact parenchymal cells, 8,600X; B) Nucleus, chromatin, nuclear membrane, mitochondria, and rough endoplasmic reticulum of liver parenchymal cell, 57,900X; C) Largemouth bass nucleated red blood cell, 22,570X; D) Another cell type found very rarely in hepatocyte isolations, a perisinusoidal, fat storing cell of Ito, 14,630X.

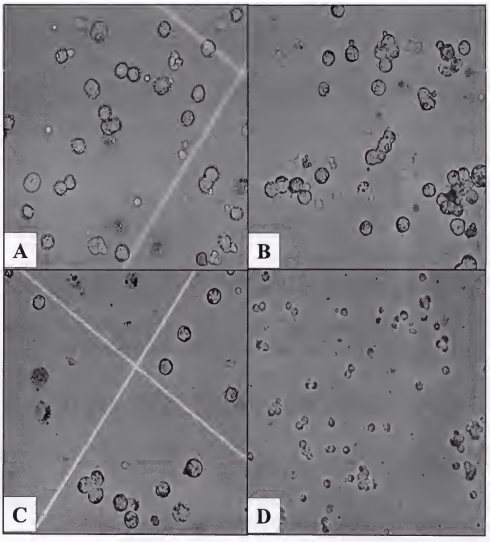

Figure 3-4. Trypan blue of isolated hepatocytes. Healthy cells exclude the blue dye, as observed out to 24 h in culture. A) 60X magnification of characteristic round hepatocytes immediately following isolation; B) 60X magnification of hepatocytes 12 h in culture; C) 60X magnification of hepatocytes 24 h in culture; D) 40X magnification used to identify and count cells prior to culturing.

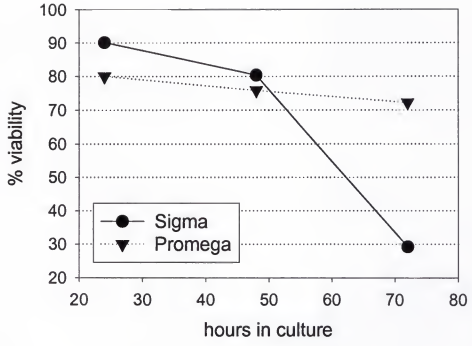

Figure 3-5. Hepatocyte viability over time. Cell viability as measured using lactate dehydrogenase leakage over time. Duplicate culture wells measured using two different kits, Sigma and Promega, illustrating variability of assay.

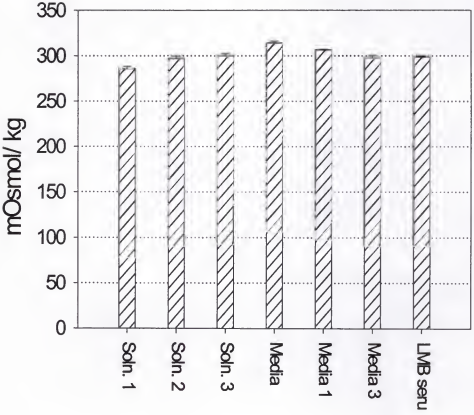

Figure 3-6. Osmolality of hepatocyte reagents. Largemouth bass primary hepatocyte media osmolality measurement relative to serum.

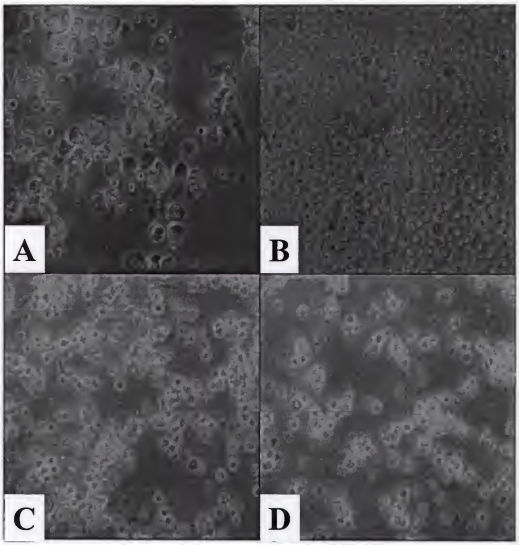

Figure 3-7. Light microscopy of cultured primary hepatocytes. A) 80X magnification of clumped hepatocytes; B) 40X magnification of characteristic round hepatocytes; C and D) 60X magnification used to identify and count cells prior to culturing.

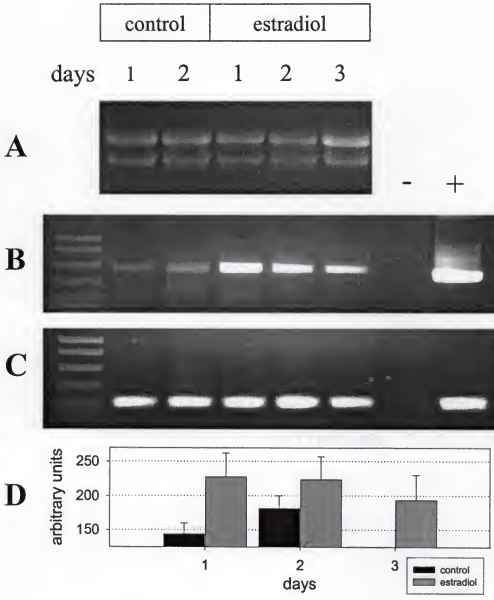

Figure 3-8. Primary hepatocyte gene expression. RT-PCR of primary hepatocyte total RNA isolated at different timepoints in control and E_2-exposed culture. Negative and positive indicate no RT present and plasmid DNA respectively. A) Ethidium bromide stained 28S and 18S rRNA bands of total RNA samples used in RT-PCR reactions; B) Vtg mRNA using degenerate Vtg primers; C) β-actin mRNA using gene-specific primers; D) Illustration of relative Vtg mRNA induction of RT-PCR bands by densitometry.

CHAPTER 4
VITELLOGENIN INDUCTION IN SHEEPSHEAD MINNOW AS AN *IN VIVO* MODEL FOR ESTROGENICITY

Introduction

All studies described in this chapter were conducted in collaboration with Drs. Michael Hemmer and Leroy Folmar at the United States Environmental Protection Agency (US EPA) Gulf Ecology Division in Gulf Breeze, FL. Kevin Kroll of the Molecular Biomarkers Lab in the Interdisciplinary Center for Biotechnology Research conducted all sheepshead minnow plasma vitellogenin assays. My research contribution was all aspects of the vitellogenin mRNA characterization and analysis.

Sheepshead minnow (SHM) is a relatively small estuarine fish native to the Atlantic and Gulf coasts of the United States. Historically it has served as a good laboratory model for examining fish biology. It is easily housed and maintained at high population densities and has a short generation time (3 to 4 months). Advantageous to studies relevant to reproduction is that sex is easily distinguishable externally (Figure 4-1). Females are usually smaller in size without a black feature on the fins. Male fish are distinguished by their black markings on their fins and larger body size. All these features are important for studying estrogen-regulated gene regulation *in vivo*. Other fish models such as killifish, rainbow trout, medaka, and fathead minnow are useful. But for our purposes SHM were previously characterized at the US EPA wet lab in Gulf Breeze, FL and provided a model to study estuarine pollution.

The mechanism of estradiol or xenoestrogen action is primarily mediated through intracellular receptors acting as transcription factors that regulate gene expression. This level of regulation results in *de novo* synthesis of mRNAs and protein that result in altered physiological responses. This is the very simplistic view of how these types of compounds affect animals *in vivo*. There are many other levels of regulation in various tissues, but it is unclear at this time what they all are and how they interact with each other. This is one of the most important reasons why *in vivo* exposures are necessary for investigating estrogenic responses.

Vitellogenin mRNA and Protein as Biomarkers of Exposure

The expression of vitellogenin (Vtg) in oviparous vertebrates, especially in males, is an excellent biomarker for compounds acting through the estrogen receptor (ER) (Denslow et al. 1999b, Heppell et al. 1995). Vitellogenin, the estrogen responsive precursor protein for egg yolk, is normally produced in the liver of mature females. Male fish also possess the Vtg gene, and although it is thought that males do not normally produce Vtg, it can be induced in response to an endogenous or exogenous estrogen (Mommsen & Walsh 1988). Male fish exposed to natural or anthropogenic estrogens in the wild produce high levels of plasma Vtg (10 – 50 mg Vtg/mL), although Vtg levels in polluted sites commonly fall in the 1 to 500 μg/mL range (Bevans et al. 1996, Folmar et al. 1996, Harries et al. 1996, Jobling et al. 1998). Quantitation of plasma Vtg in males using monoclonal antibodies to Vtg is a good measure of exposure to environmental estrogens (Denslow et al. 1997b, Denslow et al. 1999b, Folmar et al. 1996). In addition to measuring Vtg at the protein level, gene activation at the molecular level can be determined by measuring Vtg mRNA expression in the liver. Vtg mRNA is a useful

biomarker at the molecular level (Bowman & Denslow 1999, Denslow et al. 1999a). While both Vtg mRNA and protein are products of the same pathway, they are distinguishable biomarkers. mRNA transcription is induced immediately, its half-life is short as it is quickly degraded in the absence of estrogen. The protein, however, is slower in forming and is also more stable to degradation. Thus, by characterizing hepatic Vtg mRNA induction, we can detect an immediate reaction to the presence of estradiol (E_2) or its mimics, while plasma Vtg tracks exposure over a longer time interval. The first hypothesis that was tested was that a high dose acute exposure to E_2 would elicit an induction of Vtg mRNA before the accumulation of plasma Vtg over time. Another hypothesis was that Vtg mRNA and protein accumulation would occur faster and with a higher average response following repeated acute exposures. These hypotheses were tested using a time course of Vtg mRNA and protein following single and double E_2-injection.

Environmental Estrogens

The endocrine disruption hypothesis presents a formidable challenge to investigating the capacity for man-made chemicals to interact with living systems. As described in Chapter 1, legislative mandates have helped motivate the testing of many xenoestrogens using various bioassays. The potency of synthetic estrogens diethylstilbestrol (DES) and ethinylestradiol (EE_2) vary with respect to estradiol depending on the assay of estrogenicity used, although EE_2 is thought to be more potent over all. Specifically, the relative potencies of EE_2, E_2, and DES are 515.7, 100, and 90% respectively as measured by competition binding assays in channel catfish (Nimrod & Benson 1997). They are interchangeably used as positive controls in many experiments.

This is a little ironic since DES has demonstrated human endocrine and reproductive toxicity, (Herbst & Bern 1981) and EE_2 is one of the most potent endocrine-disrupting components in municipal effluents (Purdom et al. 1994).

In the studies presented in this chapter, we investigated the estrogenic effects of E_2, EE_2, DES, nonylphenol (NP), methoxychlor (MXC), and endosulfan (ES) on SHM Vtg. NP is the primary degradation product of alkylphenol ethoxylates. This class of compounds was discussed earlier in Chapter 1. The bioconcentration factors for nonylphenol range from 0.9 to 1250 in various fish species (Servos, 1999). NP is estrogenic by virtually all bioassays tested including *in vitro* ER-binding, expression assays, and the *in vivo* mouse uterotropic assay (Petit et al. 1997, Shelby et al. 1996, Soto et al. 1995, Vonier et al. 1996). Previous NP data have also shown *in vivo* plasma Vtg induction in fish (Arukwe et al. 1998, Christiansen et al. 1999, Madsen et al. 1997).

Methoxychlor and endosulfan are organochlorine pesticides that are sometimes classified as estrogen and sometimes not, depending on the assay. MXC and ES have both induced proliferation of estrogen-dependent MCF-7 human breast cancer cells (Soto et al. 1995) suggesting that they are both estrogen agonists. An estrogenic response however, was not seen for MXC in other various *in vitro* assays including ER binding (Shelby et al. 1996, Vonier et al. 1996). Nevertheless in the *in vivo* mouse uterotropic assay MXC has tested positive as an estrogen (Shelby et al. 1996). The apparent reason for these assay discrepancies is that MXC requires metabolic conversion to active demethylated metabolites which bind the estrogen receptor (Bulger et al. 1978, Schlenk et al. 1998). This is a classic example of why *in vitro* assays must always be verified by *in vivo* experiments.

Endosulfan can induce transcriptional activation of trout ER-transfected yeast cells and of Vtg mRNA in trout hepatocytes (Petit et al. 1997). In other *in vitro* assays and the *in vivo* mouse uterotrophic assay, ES was not estrogenic (Shelby et al. 1996, Vonier et al. 1996). Part of the reason for choosing these particular chemicals for the SHM *in vivo* studies was to help resolve some of these discrepancies presented among other assays.

The aqueous exposure experiments presented in this dissertation tested the hypothesis that estrogens and proposed estrogen mimics would exhibit dose- and time-dependent up regulation of Vtg mRNA with concomitant increase in plasma Vtg. Specifically, synthetic and natural estrogens would induce transcription faster than NP, MXC, and ES and be approximately 100 to 1000-fold more potent. Another hypothesis tested was that E_2-induced Vtg mRNA and protein would be removed be much faster than that induced by NP following transfer to clean water.

Experimental Objective

Two approaches to investigate the relationship between Vtg mRNA and plasma Vtg protein are to select different routes and duration of chemical exposure. Acute exposures provide an important framework to test rate of mRNA and protein induction and to characterize how long-lived these species are in the absence of continued exposure. This can mimic pulses of hormones in the body, or pulses released into the environment. Often environmental exposures are not pulsed, but rather are constant. Therefore chronic or constant aqueous exposure can provide evidence to how organisms adapt to exposures at the mRNA and protein level. Both routes and durations of exposure can be used to evaluate the dose and timing of how environmental contaminants may

affect gene expression. The objective of these SHM studies was to test the *in vivo* response to estradiol and estrogen mimics. These experiments using different routes of exposure over dose and time would be used to characterize SHM as a useful *in vivo* bioassay for estrogenic activity. All of these experiments would provide important information on the sensitivity of Vtg mRNA and protein as biomarkers, as well as understanding some of the basic endocrinology behind hormone-induced gene expression.

Materials and Methods

Fish Collection and Maintenance

Acquisition and maintenance of all fish were completed by the staff at the Gulf Ecology Division at the US EPA in Gulf Breeze, FL. Sheepshead minnows (*Cyprinidon variegatus*) were either purchased from CD Associates, Inc. (Baton Rouge, LA), or collected by bag seine from the Santa Rosa Sound, FL. Fish were acclimated in aerated all-glass aquaria under constant conditions of 25 +/-2° C and 18 to 22 part per thousand salinity for a minimum of two weeks prior to exposures. Dissolved oxygen (mean 6.3, range 5.3 – 7.7 mg/L) and pH (range 7.5 – 8.0) were measured at the beginning and at each sampling period. The fish were maintained on a 16L/8D photoperiod, and fed Tetramin flakes twice daily. All aqueous exposure conditions followed ASTM (1988) guidelines for flow-through aquatic toxicant exposures with fish.

Experimental Exposures

The first set of experiments consisted of two separate injection protocols using 17β-estradiol. In the first experiment, 25 fish were injected with 15 μL of 2 mg/mL E_2 (approx. 5 mg/Kg body weight) in triethylene glycol (TEG). The fish were returned to the aquaria, and sampled in groups of 5 fish at 4, 8, 24, 48, and 72 h following the injection. Five male control fish were sampled at time zero. In the second experiment, thirty-five fish were treated as described in experiment one. Five fish were sampled at 4 days post injection, with the remaining fish injected a second time with 15 μL of 2 mg/mL E_2 in TEG. Five fish were sampled at 1, 3, 6, 9, and 12 days following the second injection. Separate control fish were injected with 15 μL TEG as vehicle controls.

The second set of experiments were continuous flow-through exposures to several doses of E_2, diethylstilbestrol (DES), ethinylestradiol (EE_2), nonylphenol (NP), methoxychlor (MXC), and endosulfan (ES). A time course of exposure and collection was conducted for each dose of each chemical. A six-cell dosing apparatus was used to dispense 1L of test solution to each experimental tank at 20 cycles/hour. Three dual channel Hamilton Microlab 500B dispensers fitted with 100 μl Hamilton syringes were used to mix chemical stock solutions at 50 μl/cycle into the apparatus mixing chambers. Calculated dilutions for each of the chemical exposures were as follows: E_2 (20, 100, 200, 500, 1000, and 2000 ng/L); DES (0.2, 2, 20, 100, and 200 ng/L); EE_2 (20, 100, 200, 500, and 1000 ng/L); NP (1, 10, 20, 40, and 80 μg/L); MXC (1.5, 3, 6, 12, and 24 μg/L); and ES (25, 50, 100, 200, 300, 600, 900, and 1200 μg/L). Triethylene glycol served as vehicle control in all experiments. Estradiol at a nominal concentration of 100 ng/L

served as a positive control for the xenoestrogen experiments. For each dose of E_2, DES, and EE_2, fish were exposed for 0, 2, 4, 7, 10, 13, or 16 days. Exposures of 0, 2, 5, 13, 21, or 35 days were conducted for the NP, MXC, and ES experiments.

The last set of experiments was to evaluate the decrease of Vtg mRNA and protein levels following 16 days of continuous flow-through exposure to E_2 and NP. The same dosing apparatus mentioned above was used to mix and control exposures in these experiments. Nominal exposure concentrations for E_2 were 100 and 1000 ng/L, and for NP were 10 and 100 µg/L. TEG served as vehicle control in all experiments. For each dose of E_2 or NP, all fish were exposed for 16 days, except those collected at days 0 and 8. Following transfer to clean water at day 16, fish were collected on days 16, 18, 20, and 24 for Vtg mRNA analysis, and days 16, 18, 20, 24, 33, 37, 42, 70, and 112 for plasma Vtg.

Blood and livers were collected from all experimental and control fish. The tail was severed at the caudal peduncle and blood was drawn using a heparinized hematocrit tube. After centrifugation for three min at 13700 x g the plasma was aspirated and stored at –70°C until analyzed. The liver was excised, then immediately flash-frozen in liquid nitrogen and stored at –70°C until analyzed.

For constant aqueous exposures, chemical concentrations were measured at regular intervals during all experiments (Table 4-1). Concentrations remained constant for the duration of all experiments. All chemicals were measured except DES. The methods used to determine chemical concentrations are unique to each chemical and have been described previously (Folmar et al. 2000, Hemmer et al. 2001). Personnel at the US EPA in Gulf Breeze, FL, conducted all chemical analyses.

RNA Isolation, Identification and Verification of Vitellogenin Sequences

RNA was isolated as described previously. (Bowman & Denslow 1999, Bowman et al. 2000) Briefly, the individual liver tissues were processed for RNA using either RNeasy kits (Qiagen) or acid phenol guanidinium-isothiocyanate. (Chomczynski & Sacchi 1987) The samples were treated with Proteinase-K, then measured at 260 and 280 nm using a spectrophotometer. The 260 nm reading was used to estimate the concentration of total RNA recovered from the isolation. The 260/280 ratio, as well as a 1% agarose-formaldehyde gel stained with ethidium bromide, were used to verify the quality of the RNA in each sample. Oligo-dT primers, dNTPs, 5X transcription buffer (Life Technologies), and Superscript II (Life Technologies) were used to reverse transcribe 2 μg total RNA. The degenerate Vtg primers (80 pmol/μl), described in Chapter 2 of this dissertation, in 10X reaction buffer containing 1.5 mM $MgCl_2$ (Perkin-Elmer), 2.5 mM dNTPs, 1U AmpliTaq (Perkin-Elmer), and 2 μl of cDNA from the reverse transcription reaction were used to amplify portions of the Vtg gene by the polymerase chain reaction (PCR). The conditions for the PCR were: hold at 80°C for 3 min; hold at 94°C for 3 min; 35 cycles of 94°C for 45sec, 52°C for 90sec, 72°C for 45sec; hold at 72°C for 10 min; and hold at 4°C for one hour. The PCR products were analyzed and purified by 1.2% agarose gel electrophoresis. The single band corresponding to the amplified cDNA was extracted from the gel and purified using Qiaquick gel extraction spin columns (Qiagen). The purified PCR products were ligated into Promega's pGEM-T Easy vector and transformed into *E.coli* (DH5α). Five plasmid clones containing the amplified fragments were picked randomly and purified for sequence determination and probe preparation. Using an ABI PRISM Dye Terminator

Cycle Sequencing Kit (Perkin Elmer) and M13 primers from the DNA Synthesis Core Facility (Biotechnology Program, University of Florida), sequencing reactions were performed on the five isolated plasmid preparations, and submitted to the DNA Sequencing Core Facility at the University of Florida for sequence determination. BLAST (Altschul et al. 1997) and multiple alignment programs of Corpet (1988) were used to analyze the Vtg cDNA sequence results. The two sequences we have identified as VIT 1 and VIT 2 show 72% nucleic acid homology and were confirmed by BLAST analysis to be vitellogenin.

mRNA Quantification

Complementary DNA (cDNA) probes were made using the cloned VIT 1 or VIT 2 fragments. Templates for each probe were cut from the plasmid vector using EcoRI. The digestion reactions were purified by gel electrophoresis and extracted using Qiaquick spin columns (Qiagen). These templates were used for the synthesis of [α-^{32}P]-labeled cDNA probes using a Strip-EZ DNA Kit (Ambion) according to manufacturer's instructions, and purified using TE-Midi SELECT-D, G50 spin columns (5 Prime-3 Prime, Inc.). A SHM β-actin probe (clone courtesy of Dr. Ronald Ferguson) was prepared as described for Vtg.

Vitellogenin mRNA was quantified as described previously. (Bowman & Denslow 1999, Bowman et al. 2000) Briefly, for Northern blot analysis, 12 μg of total SHM liver RNA was denatured and separated on a 1% agarose-formaldehyde gel. The RNA was transferred to a nylon membrane (Biodyne B; Life Technologies) using downward capillary action, followed by UV-crosslinking using a Stratalinker 1800 (Stratagene). Nylon membranes were stained with methylene blue to verify successful

transfer and even loading of the samples (Herrin & Schmidt 1988). Membranes were pre-hybridized in a glass cylinder using a Techne Hybridiser oven with ExpressHyb hybridization buffer (Clontech) for 30 min at 68°C. The membranes were then incubated at 68°C for one hour in fresh hybridization buffer containing approximately 3 x 10^5 dpm VIT 1 or VIT 2 probe/mL. For the time course and water exposure experiments, the SHM VIT 1 probe was utilized. The nylon membranes were then washed twice with 2X SSC, 0.1% SDS for 20 min at 25°C, then twice with 0.1X SSC, 0.1% SDS for 30 min at 60°C or 68°C. The nylons were then wrapped in saran wrap and exposed to BioMax MR X-ray film (Eastman Kodak) for visualization and were also exposed to a phosphorscreen for image quantification using a PhosphorImager (Molecular Dynamics, Inc.).

For the quantification of Vtg mRNA, sense Vtg cRNA was transcribed *in vitro* from the vector using a Megascript Kit (Ambion) according to the manufacturer's suggestions. Sense Vtg cRNA was analyzed by gel electrophoresis and quantified by spectrophotometry. Concentrations from 0.1 ng to 5 µg Vtg cRNA serving as standards were denatured and loaded onto a Biodyne B nylon membrane using a slot blot apparatus (Schleicher and Schuell). Twelve µg of sample and standard RNAs were denatured in denaturing buffer (containing 20X SSC, formamide, and formaldehyde) and loaded into slots on the same membrane. The nylon membrane was stained with methlyene blue and hybridized as described above for Vtg mRNA quantification. To standardize gene expression across individuals, Northern and slot blots were stripped and re-probed with SHM β-actin. All sample values obtained were corrected individually to β-actin levels, except with the DES and EE_2 time course. Data was collected using a PhosphorImager as

described above. Actual Vtg mRNA values reported were calculated from a standard curve generated from the synthesized Vtg cRNA standards.

Differential Display RT-PCR

Differential display reverse transcription-polymerase chain reaction (RT-PCR) was performed with the RNAimage mRNA Differential Display system (GenHunter) using one-base anchored oligo-dT primers (Liang et al. 1994). This work was completed in collaboration with the Differential Display Core facility at the University of Florida Cancer Center. DNase-treated total RNA (0.2 μg), isolated from control or treated sheepshead minnow livers were reverse transcribed using 0.2 μM of anchor primer and 100 U MMLV reverse transcriptase in a total volume of 20 μL as described by the manufacturer. For each condition, we used three separate liver samples to be able to distinguish false positives. PCR reactions (20 μL) were performed following the RNAimage protocol and included one-tenth volume of the reverse transcription reaction, 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 μM anchor primer and arbitrary primer, 2 μM dNTPs, 2.5 μCi -[^{33}P]-dATP (2000 to 4000 Ci/mmol), and 1 U AmpliTaq DNA polymerase. After an initial denaturing step of 94°C for 5 min, 40 PCR cycles were run with the following conditions: 94°C for 15 sec; 40°C for 2 min; 72°C for 30 sec; followed by a 10 minute 72°C extension step. Aliquots of each PCR reaction were heated for 3 min at 80°C with DNA sequencing loading dye and separated by electrophoresis on 5% denaturing Long Ranger gels. The gels were dried under vacuum at 80° C and exposed to Biomax MR X-ray film for 18 to 48 hs.

Bands of interest were located on the gel, cut out, and then soaked in 100 μl of ddH_2O for 10 min, followed by boiling for 20 min to elute the DNA. A fraction of the

material was used as template with the same primer pairs to reamplify the DNA. A 40 µl reamplification reaction was prepared for each cDNA with arbitrary primer 23 (2 µM) and the anchor G-T_{11} primer (2 µM). The PCR conditions were set up as follows: 80°C for 3 min → 95°C for 2 min → 40 cycles of 94°C for 15 sec, 40°C for 2 min, 72°C for 30 sec → 72°C for 10 min → 4°C soak. Twenty microliters of the PCR samples were run on a 1% agarose gel and stained with ethidium bromide. If the size of the re-amplified product was correct, the cDNA band was gel purified, cloned and sequenced as described above.

Protein Analysis

The presence of Vtg in serum samples was verified by electrophoresis using Tris-tricine gels and Western blot analysis as described previously (Denslow et al. 1997b). All plasma samples were diluted 50-fold in 1X Laemmli sample buffer and 10 µl were applied to separate wells of 7.5 % polyacrylamide gels (~0.2 µl plasma per well). Samples of purified sheepshead minnow Vtg (0.1 and 0.5 µg) were included in separate wells as positive controls. Multimark molecular weight markers (Novex) were used to calibrate the gels. The gels was electroblotted to PVDF membranes (Immobilon-P-Millipore) in 10 mM MES (morpholino ethane sulfonic acid), 10% methanol, 0.01% SDS, pH 6.0, overnight at 20 V and 4°C. For Western blot detection, the membrane was blocked with 5% nonfat dry milk in TBSTZ (10 mM Tris, 150 mM NaCl, 0.05% Tween, 0.02% sodium azide, pH 7.2) at 25°C for two hours. The blot was incubated with primary monoclonal antibody HL 1080 1C8-3C11 in blocking solution, developed with a secondary goat-anti-mouse alkaline phosphatase-linked antibody (Pierce) and developed

with bromochloroindoyl phosphate/nitro blue tetrazolium as described previously (Denslow et al. 1997b).

A direct ELISA (enzyme-linked immunosorbent assay) was used to quantify plasma Vtg as described previously (Denslow et al. 1999b, Folmar et al. 1996, Folmar et al. 2000) using avidin-biotin complex reagents (Pierce). The overall sensitivity of the ELISA used in this study was 2 μg/mL for plasma Vtg. The assay itself is more sensitive by a factor of two hundred when pure Vtg is used. We are able to measure 0.5 ng Vtg per ELISA well in a volume of 50 μL. The lower sensitivity for plasma samples is due to the requirement to dilute plasma 1:200 to eliminate interferences. The linear portion of the standard curve extends from 0.01 to 0.8 μg/mL purified Vtg. All of the standard curves were prepared with added control male plasma, diluted to the same concentration as the samples, so that the samples and standards have the same composition. Four different standard curves are were performed with male plasma diluted to 1:200 (for the samples containing the least amount of Vtg), 1:10,000 (for intermediate samples), 1:100,000 and 1:million (for the highly induced samples). On occasion a sample will have a vitellogenin concentration that is outside these standard curves. In that event, the assay is repeated with the appropriate standard curve. The primary antibody used was HL 1330 (5C9-4A8) raised against sheepshead minnow Vtg. The plate coated with samples and standards was incubated with the monoclonal antibody overnight at 4°C in a humidified chamber. For the rest of the assay the following reagents from Pierce were used: goat-anti-mouse IgG (H&L), biotin, and the streptavidin alkaline phosphatase conjugate. After washing the plate, Vtg was quantified colorimetrically with the alkaline phosphatase substrate, *p*-nitro phenyl phosphate (in carbonate buffer with 2 mM $MgCl_2$,

pH 9.6) at 405 nm in an ELISA plate reader. All samples and standards were run in triplicate. The coefficients of variation and correlation coefficients for this assay were <10% and > 0.95% respectively.

Results

Vitellogenin I & II Cloning, Sequencing and Characterization

To clone a segment of the Vtg mRNA from SHM liver, Vtg primers designed to a conserved region near the C-terminus of vitellogenins found in Genbank (Benson et al. 1999) were used (previously described in Chapter 2 of this dissertation). These degenerate primers amplified a ~460 bp fragment from SHM liver RNA (Figure 4-2). The size of the amplified SHM fragments correspond to the expected size based on fish Vtg sequences in the database. SHM liver RNA samples from different exposure groups used in parallel RT-PCR reactions gave the same size fragment. The intensity of the bands correlated with the treatment conditions, i.e., more intense bands were observed in the lanes corresponding to high doses of E_2 and EE_2 (Figure 4-2).

Because the cloned SHM Vtg fragments were initially obtained by PCR, five independent clones in both orientations (both strands) were sequenced. Sequence analysis revealed two sequences, termed VIT 1 and VIT 2, with 72% identity (Genbank accession numbers AF239720 and AF239721 respectively). A BLAST search (Altschul et al. 1997) of each showed specific homology to most of the vitellogenin sequences in the database. As shown in Figure 4-3, the two SHM Vtg fragments VIT 1 and VIT 2 correspond to the VIT 1 and VIT 2 (Genbank accession numbers Q90508 and Q98893) of *F. heteroclitus*, respectively (LaFleur et al. 1995a, LaFleur et al. 1995b).

Both SHM VIT 1 and VIT 2 cDNAs were used in Northern blots to determine the size and number of mRNA bands that could hybridize with these probes at increasingly stringent (higher temperature) wash conditions. Extracted hepatic total RNA from male SHM injected with ~5 mg estradiol/Kg disclosed 3 bands, a major band at 5.0 kb and two minor bands at 3.3 and 1.7 kb (Figure 4-4A), as determined using Promega's RNA ladder. There was no band detection in lanes containing RNA from unexposed fish when probed with either VIT 1 or VIT 2, even at low stringency (50°C) wash conditions, suggesting that none of the three bands are present in male control fish in sufficient levels for detection by Northern blot. The absence of signal in the control lanes also suggests that the VIT 1 and VIT 2 probes do not crossreact with rRNAs, which migrate in the gel close to the smaller sized bands. The blot reprobed for β-actin mRNA showed that consistent amounts of total RNA were applied to the gel for both control and E_2-treated samples (Figure 4-4B).

To determine whether the three bands hybridized specifically, with either VIT cDNA probes, the Northern blots were washed at increasing temperatures, 60°C followed by 68°C (e.g., increasing the stringency). Higher temperature washes (>60°C) are normally used to remove nonspecific binding (Sambrook et al. 1989). After washing at 68°C, all three bands persisted on both blots. The data shown in Figure 4-4C is the relative quantification, by PhosphorImager, of the bands shown in Figure 4-4A at the two temperatures and corrected for β-actin levels. The response for each band was normalized as a percent of the total response for each temperature condition since the absolute band intensity decreased with increased stringency. Figure 4-4C indicates VIT 1 and 2 probes bound differently to the three bands, with VIT 1 binding relatively more

efficiently to the 3.3 and 1.7 kb bands. The VIT 2 probe binds better to the intact 5 kb band.

Time Course of Vitellogenin Induction Post Injection

Two injection experiments were performed. In the first experiment, male SHM were injected with a single dose (5 mg/Kg) of E_2 and sampled three times in the first day (at 4, 8, and 24 h), and then at days 2 and 3, as described. Using a slot blot method to quantify the amount of Vtg mRNA, 0.04 ng/μg total RNA was detected in untreated males, this was found consistently in all of the reported SHM experiments. This amount increases to 0.2 ng/μg total RNA 4 h after the single injection, a 5-fold induction. After 48 h, Vtg mRNA reaches a maximum of 1.4 ng/μg total RNA, representing a 35-fold induction over background. Within seventy-two hours, the level dropped back to 0.2 ng/μg total RNA, the level observed at 4 h (Figure 4-5A and 4-5C).

In the second injection experiment, SHM were treated with two injections of 5 mg/Kg of E_2 four days apart. Immediately prior to the second injection (labeled day 0 in Figure 4-5D and 4-5F), Vtg mRNA levels were measured at 0.2 ng/μg total RNA. This level correlates well with the amount observed for the 72 h time point in the single injection experiment (Figure 4-5C). Twenty-four hours following the second injection, Vtg mRNA increased to 0.5 ng/μg total RNA, 11-fold over controls. The maximum response measured post second injection was 0.65 ng/μg total RNA at 72 h, which represents a 16.2-fold induction over controls. This level is approximately 3 times higher than observed for the 72 h time point following the single E_2-injection. Within 6 days post second injection, Vtg mRNA levels had returned to baseline. Unfortunately, no

fish were sampled 48 h after the second injection, where the maximum level Vtg mRNA induction was expected to occur.

Both Western blots and ELISAs were used to measure plasma Vtg samples from the same fish used for RNA analyses (Figure 4-6A and 4-6B). Western blot analysis of the single E_2-injected fish revealed the induction of two bands at 180 and 160 kDa, which appear in the plasma within 24 h after the single injection (Figure 4-6A), both in approximately the same time frame. The two bands persist through the entire time course. Large lipoglycoproteins, such as Vtg, are difficult to transfer evenly to membranes for Western blotting (Xu & Shively 1988). Even though we added 0.01% SDS in the transfer buffer there was still indiscriminate variation among samples (Figure 4-6A). Therefore we quantified the amount of plasma Vtg at each time point by ELISA (Figure 4-5B). Vitellogenin levels increased from undetectable at time 0 to 3.2 mg/mL by 24 h, and reached a maximum of 15.7 mg/mL within 48 h.

In the double estradiol-injection experiment, Western blot analysis revealed that plasma Vtg levels prior to the second injection were equivalent to that observed after three days with the single injection experiment (Figure 4-6C). Following the second injection, the levels of plasma Vtg increased within the first 24 hr, then appeared to decrease and level off by 6 days (Figure 4-6C). Both Vtg bands persisted in the plasma over the entire time course, although the 180 kDa band appeared to degrade faster than the 160 kDa band after 9 days. There was, however, no observable smearing or presence of smaller bands. An alternative evaluation is a more rapid turnover of the corresponding mRNA species. ELISA supported results of the Western blots. There was a maximum average accumulation of 40 mg Vtg/mL by 1 day after the second injection (Figure 4-

6D). Plasma Vtg then decreased and leveled off at approximately 20 mg/mL for the duration of the experiment.

Vitellogenin Dose and Time Response with Constant Aqueous Exposure

Multiple constant aqueous exposures to estradiol and xenoestrogens were conducted from 1997 to 2000. Livers and plasma samples were collected and analyzed for Vtg mRNA and plasma Vtg protein levels. A subset of the liver samples (100 and 1000 ng E_2/L, 200 and 2000 ng EE_2/L) following seven days exposure were analyzed by Northern and slot blot analysis, and the plasma was analyzed by ELISA (collaboration) (Figure 4-7). These Northerns showed no detectable levels of Vtg mRNA in controls, whereas in fish exposed to both E_2 and EE_2 there were significant amounts of Vtg mRNA in the liver (Figure 4-7A). The same three bands observed with the E_2-injected fish were detected using the SHM VIT 1 as a probe. The nylon membranes were re-probed for β-actin mRNA (Figure 4-7B) to control for even loading of total RNA. Data presented in Figure 4-7A and 4-7B are uncorrected for loading levels and are therefore more qualitative in nature. The induction of Vtg mRNA was also quantified by slot blot analysis (Figure 4-7C). A low level of 0.04 ng Vtg mRNA/μg total RNA was measured in control males. Treatment of fish with E_2 or EE_2 for 7 days under a constant exposure regimen increased the levels of Vtg mRNA over that of unexposed control fish (Table 4-2). There were statistically significant increases ($P<0.05$) in Vtg mRNA with the high doses of both E_2 and EE_2 compared to the low doses or to controls. The apparent increase at the low doses over controls was not statistically significant (200 ng E_2/L- $P=0.122$, and 100 ng EE_2/L- $P=0.128$) probably due to small sample size.

ELISA results for this subset of samples demonstrate that plasma Vtg accumulates to very high levels by 7 days of constant exposure to high doses of either chemical (Figure 4-7D). This induction is much higher than that seen for the injection experiments, even though in those experiments fish were injected with high doses of E_2. After 7 days, there were 18.4 mg/mL plasma Vtg for fish treated with 200 ng E_2/L, but this level was 63.4 mg/mL for fish treated with 2000 ng/L, representing a 3.5-fold difference. For fish treated with EE_2, plasma Vtg levels were 15.5 mg/mL for 100 ng/L and 57.4 mg/mL for the 1000 ng/L treatment. This represents a 3.7-fold difference in plasma Vtg, showing the dose responsiveness of this treatment. Plasma Vtg was significantly increased with the high dose of each chemical compared to the respective low dose exposure or control ($P<0.05$). (Figure 4-7D) The obvious induction of Vtg at the low doses was not statistically significant with the small sample number (200 ng E_2/L- $P=0.092$, and 100 ng EE_2/L- $P=0.0554$).

Gene regulation by estradiol, diethylstilbestrol, and ethinylestradiol

In collaboration with the EPA lab in Gulf Breeze, FL, SHM were exposed over 16 days to varying doses of E_2, DES, and EE_2 in the water (Figures 4-8 and 4-9) (Folmar et al. 2000). At twenty nanograms per liter, only DES induced Vtg mRNA and protein over background albeit not until after 7 days of constant exposure. The lower DES doses (or the 20 ng/L doses of E_2 and EE_2) did not however up regulate Vtg mRNA or protein. All of these treatments at or above 100 ng/L induced Vtg mRNA by the first collection, at 2 days of constant exposure, and by day 4, plasma Vtg was observed. The fish from the E_2-treated fish up regulated Vtg mRNA, with a high variability in the response relative to the dose (Figure 4-8A). There was a clear dose response of Vtg mRNA in fish exposed to EE_2 and DES (Figure 4-8B and 4-8C). Because of the high variability in response, the

rates of plasma Vtg accumulation were not significantly different in the four highest E_2 or EE_2 exposure concentrations, although these did result in significantly greater amounts than the 20 ng/L treatments.

Differential display RT-PCR was used to identify gene induction fingerprints to these potent estrogens (Denslow et al. 2001). As Figure 4-10 demonstrates, DES and EE_2 both appear to induce the same suite of mRNAs as E_2 following exposure. When compared to the control samples, all three potent estrogens have practically the same gene induction fingerprint. With this technique it is possible to excise a band of interest, clone it, and sequence it to verify its identity. This cloned fragment can also be used to verify the type of gene regulation seen on the gel. Two of these fragments were identified, by sequencing, as zona radiata protein 2 and transferrin. The cloned ZP2 cDNA fragment hybridized to a SHM Northern blot, validating E_2 up regulation of this gene in SHM (Figure 4-11).

Vitellogenin induction by nonylphenol, methoxychlor, and endosulfan

SHM Vtg mRNA and protein were also quantified following exposure to different concentrations of NP, MXC, or ES over time (Figures 4-12 and 4-13) (Hemmer et al. 2001). Vitellogenin mRNA in fish exposed to the lowest nominal concentrations of NP and MXC (1.0 and 1.5 μg/L respectively) was not significantly induced over background. At these lowest chemical concentrations, plasma Vtg was detected late in time (21 days of NP and 42 days of MXC) relative to the other doses, but showed significantly lower amounts of Vtg accumulation (5 mg/mL and 1.6 mg/mL respectively) as compared to higher doses (see below). The rest of the exposures (> 10 μg/L NP and > 3 μg/L MXC)

led to dose dependent induction of Vtg mRNA, and a linear increase in plasma Vtg accumulation.

For nominal doses of 10 to 80 μg/L of NP the accumulation rate and plateau of Vtg mRNA and protein was faster and higher with increasing dose (Figures 4-12A and 4-13A). By five days of exposure to NP, Vtg mRNA was induced up to 152, 565, 2360, and 5621 pg/μg total RNA and plasma Vtg up to 2.4, 13.1, 27.6, and 30 mg/mL (10, 20, 40, and 80 μg/L respectively). The induction of Vtg mRNA and protein by the different doses of MXC was similar but with a slight delay in reaching a plateau level (Figures 4-12B and 4-13B), as best observed at the lowest effect dose (3 μg/L) at 21 days. With five days of constant MXC exposure, Vtg mRNA was induced up to 114, 673, 1019, and 11366 pg/μg total RNA and plasma Vtg up to 0.01, 2.6, 14.1, and 20.2 mg/mL (3, 6, 12, and 24 μg/L respectively). As best illustrated at the mRNA level, the overall plateau levels were close to that observed at 5 days of NP exposure, but with MXC were slightly delayed until 10 to 13 days of exposure. This delay is probably due to the metabolism of MXC to the estrogen-active compound. At all exposures tested 25 to 1200 ng/L, ES showed no detectable Vtg mRNA induction or plasma Vtg accumulation in SHM.

At the higher doses of each chemical there was significant mortality. Over the course of this experiment, NP at 40 and 80 μg/L resulted in 28 and 19% mortality respectively. There were 38 and 75% mortality in fish from 12 and 24 μg MXC/L, respectively. Therefore, because of acute mortality at some doses, some time course sampling was incomplete at later time points. For endosulfan, there was less than 9% mortality for all doses except 900 and 1200 ng/L which had 41 and 59% respectively.

Decreased vitellogenin levels following transfer to clean water

The decrease of Vtg mRNA and protein levels were investigated following 16 day exposure to two doses each of E_2 and NP (Figures 4-14 and 4-15). The rate and amount of Vtg mRNA accumulation for both doses of both chemicals plateaued to predicted levels by 16 days. For estradiol, the 1000 ng/L exposure resulted in Vtg mRNA steady state levels 3.5-fold above the 100 ng/L dose level at 16 days. The difference in the induction of Vtg mRNA by the two doses of NP (10 and 100 μg/L) at 16 days was approximately 14.7-fold. Both doses of each chemical had compound-specific patterns of decreased Vtg mRNA levels following transfer to clean water.

Estradiol induced vitellogenin mRNA to 1370 and 4800 pg/μg total RNA (100 and 1000 ng/L respectively) by 16 days constant aqueous exposure (Figure 4-14A). Vitellogenin mRNA levels remained high for two days following transfer to clean water (958 and 965 pg/μg total RNA). Only after 4 days did levels drop quickly, 20 pg/μg total RNA (100 ng/L) and 53 pg/μg total RNA (1000 ng/L). After 8 days of clean water following E_2-exposure, Vtg mRNA levels had returned to baseline. Plasma Vtg was induced by E_2 to maximum dose-specific levels at day 18 (Figure 4-15A). The plasma Vtg accumulation induced by 1000 ng E_2/L was undetectable following 96 days in clean water. At 100 ng/L of E_2, maximally induced plasma Vtg levels remained for 8 days, after which the levels began to slowly decline. It wasn't until 96 days in clean water following E_2-exposure, for both doses, that Vtg was finally undetectable in the plasma.

The scenario of decreased Vtg mRNA levels following nonylphenol exposure was much different than estradiol (Figure 4-14B). The 100 μg/L dose of NP increased Vtg mRNA rapidly to 9300 pg/μg total RNA by 16 days constant aqueous exposure. The

10 μg/L dose peaked at 630 pg Vtg mRNA/μg total RNA by that time point (~14.7-fold difference). As opposed to estradiol, Vtg mRNA levels induced by NP decreased to 60 and 666 pg/μg total RNA (10 and 100 μg/L respectively) after two days in clean water, approximately 10.5 and 13.9–fold drop respectively. Also unlike the estradiol-induced samples, Vtg mRNA from both doses of NP had not returned to baseline following 8 days in clean water. Specifically, Vtg mRNA levels were two (10 μg/L) or three (100 μg/L) times above background after 8 days in clean water. Plasma Vtg was induced to maximum levels after 16 days exposure to either dose of NP (Figure 4-15B). Vitellogenin induced by the high dose of NP remained until 17 days in clean water, when levels began to slowly drop off. It wasn't until 96 days in clean water that Vtg induced by the high dose was undetectable in the plasma. The 10 μg/L dose of NP maintained relatively consistent Vtg levels (25 to 35 mg/mL) for 17 days in clean water, then was undetectable by 21 days.

Discussion

Fish are exposed to contaminants in primarily two ways, in the water and through the diet. Aqueous exposures are probably the more relevant route of exposure for small fish like SHM. Detectable levels of estradiol, ethinylestradiol, and nonylphenol in rivers and streams support this route of exposure (Desbrow et al. 1998; Snyder et al. 1999). Acute (single) exposures are different from this type of constant aqueous exposure, but they can serve as a useful tool to better understand the set of molecular events (mRNA and protein) over time following an exposure. Single exposures also represent pulsed or one-time events that can be relevant since fish swim and are not always contained to an

exposed area. Therefore, both acute and constant aqueous exposures were evaluated in this SHM model.

Following the cloning and preparation of SHM Vtg cDNAs, the time course of Vtg induction following single or double E_2-injection was investigated. Dose and time response studies were conducted in animals exposed constantly to E_2 and potential xenoestrogens in water. To better understand the temporal sensitivity of aqueous exposures, decreased Vtg mRNA and protein levels following E_2 and NP washout were studied at two different doses. Although aspects of both transcription and translation were completed in all these experiments, I will be primarily discussing the results at the mRNA level, which was my primary contribution to this work.

Using the degenerate Vtg primers described in Chapter 2 of this dissertation, two Vtg mRNA sequences were cloned from SHM corresponding to the two sequences in the mummichog (*Fundulus heteroclitus*) (LaFleur et al. 1995a). The two separate genes for Vtg in mummichog (LaFleur et al. 1995b) and now SHM suggest that fish have multiple Vtg genes like frogs (Wahli et al. 1981) and chicken (Evans 1988, Wang et al. 1983). Recently, Matsubara and Ohkubo have reported that there are two Vtgs in barfin flounder (*Verasper moseri*), each with distinct roles (Matsubara et al. 1999). They suggest that one protein serves as the main nutrient for the developing oocyte, and the other is responsible for regulating water uptake in eggs during final maturation.

Due to the high degree of identity (72%) between SHM VIT 1 and VIT 2, it was important to evaluate the specificity of our probes for the 3 induced mRNA bands observed by Northern blot (Figure 4-4A and C). Higher temperature, lower salt, longer washes, and more frequent washes can be used to increase the specificity of probe-RNA

interactions. In my experience, the best way to increase the specificity and to remove non-specific binding is to increase the temperature of the wash solution. Even with multiple washes at 68° C, all three bands were disclosed with both probes, suggesting that the interaction is specific and therefore all three must contain a portion of Vtg mRNA (Figure 4-4A).

The largest and most intense band, at 5.0 kb, is of sufficient size to contain the entire predicted coding sequence for SHM Vtg (160 to 180 kDa). RNA size markers (Promega), 28S rRNA (4.0 kb) and 18S rRNA (1.9 kb) were utilized to calibrate the blots and determine the SHM Vtg mRNA sizes. SHM Vtg appears smaller than the reported sizes for *Salmo gairdneri* (6.6 kb) (Le Guellec et al. 1988) and *Oreochromis aureus* (6.5 kb) (Lim et al. 1991). Size calibration techniques, however, may account for some of the apparent species difference in Vtg mRNA sizes. Both the VIT 1 and VIT 2 probes hybridize to the same 5.0 kb band suggesting that the two full length mRNAs are similar in size, which is comparable to that seen in *Fundulus heteroclitus* (LaFleur et al. 1995b). The two minor bands (3.3 and 1.7 kb) are too small to contain the entire predicted coding sequence for Vtg. These smaller bands may be Vtg mRNA fragments, alternative splice variants or highly similar mRNAs that contain sequences that are homologous to our probes. Belonging to the apolipoprotein gene family (Wahli et al. 1981), Vtg may share significant homology in some regions with other proteins in the family. Other studies report similar smaller transcripts. At least 4 small putative Vtg transcripts at 3.8, 3.1, 1.9, and 1.3 kb have been reported in several fishes from the family Cichlidae (Lee et al. 1992).

Acute Estradiol Exposures

When the levels of Vtg mRNA are compared in fish stimulated once or twice by E_2-injection, different results are seen. Uninjected fish had very low levels of Vtg mRNA, approximately 0.04 ng/μg total RNA, as measured by slot blot (Figure 4-5C and 4-5F). While Vtg mRNA was not visible on Northern blots of control fish livers, bands were detectable within 4 h after a single E_2-injection. The levels of the mRNA continue to increase at the twenty-four hour time point. This level is similar to that measured 24 h after the double E_2-injection experiment, suggesting that the initial induction pattern is similar. After forty-eight hours, in the case of the single E_2-injection experiment, Vtg mRNA levels increased to 1.4 ng/μg total RNA, a value similar to that reported for rainbow trout (*Oncorhynchus mykiss*) (Pakdel et al. 1991). Unfortunately, we did not take samples at the same time point for the fish injected twice with E_2. The main difference between the observed results in the two E_2-injection protocols occurred at three days post single or double injection.

In the fish injected once with E_2, the levels of Vtg mRNA fell from 1.3 to 0.2 ng/μg total RNA between the second and third days, while in the twice injected fish, the levels of Vtg mRNA were at 0.65 ng/μg total RNA (the highest measured level) on the third day, suggesting that the mRNA might have been temporarily stabilized as compared to fish injected once. A study in rainbow trout supports the trend of our time course, albeit at a 10-fold lower level (Le Guellec et al. 1988). Using male rainbow trout, Le Guellec injected 3 mg E_2/Kg and observed an increase in Vtg mRNA to 0.026 ng/μg total RNA after 48 h, followed by a return to baseline values by 6 days after the single injection. Both these results differ from another study, also in rainbow trout, in which

there is a leveling off of Vtg mRNA at three days post injection (1.5 mg E_2/Kg) followed by a steep increase to >20 ng/μg total RNA at six days post injection (Pakdel et al. 1991). The widely varying results among fish species suggest that Vtg induction and kinetics may be species-specific.

Experiments with *Xenopus laevis* first showed a higher induction of Vtg mRNA from two consecutive acute exposures to E_2 (the "memory effect") than from a single exposure (Baker & Shapiro 1978). There is evidence suggesting that the "memory effect" may be due to one or more factors, such as increased transcription rate (Brock & Shapiro 1983), chromosomal re-arrangement (Burch & Evans 1986), or other mechanisms affecting synthesis, processing, export, or stability of the RNA (Tata 1976). The physiological relevance of the memory effect has yet to be established. These data in SHM support the hypothesis that repeated exposure to E_2 may increase Vtg mRNA half-life and stability as shown by the increased steady state levels found after 3 days post double injection compared to single injection.

At the protein level, there is an accumulation of plasma Vtg following the second E_2-injection. It is not known whether the 180 and 160 kDa bands represent protein products from the two different Vtg mRNAs, however, their synchronized expression would suggest this. We saw an increase and persistence of plasma Vtg for the twice-injected fish as compared to the single injected fish. This is similar to the "memory effect" for Vtg protein reported for *Xenopus laevis* (Wallace & Jared 1968). Vtg protein appears to last much longer in the plasma than the mRNA in the liver, probably because male fish lack well-developed pathways of elimination for the protein. Vtg is degraded by proteases in the plasma and is subsequently removed by the kidney. Excessive

plasma Vtg stimulation in male fish has been linked to significant kidney pathology (Folmar et al. 2001, Herman & Kincaid 1988).

To simulate a more natural route of chemical uptake, we also treated male SHM with estradiol, diethylstilbestrol, ethinylestradiol, nonylphenol, methoxychlor and endosulfan in separate flow-through aquatic exposures. These experiments were conducted using a large range of doses over varying lengths of time up to 42 days. For exposure route comparison, a subset of these samples (100 and 1000 ng E_2/L, 200 and 2000 ng EE_2/L following seven days exposure) was analyzed by Northern and slot blot analysis. The Vtg mRNA levels induced by the lower doses of E_2 and EE_2 are similar to those observed 48 h after a single injection of 5 mg E_2/Kg. The high aquatic dose of E_2 or EE_2 (2000 and 1000 ng/L, respectively), however, significantly induced Vtg mRNA [119 (E_2) and 16.3 (EE_2) ng/μg total RNA] beyond that observed with the injection experiments.

The observations made for E_2 suggest that constant exposure to chemicals in water, even at lower doses, appears to stimulate the estrogen receptor pathway more than a single or double injection with a high dose. The grounds for comparing an injection vs. aqueous exposure assumes that by seven days an equilibrium would be established between the water dose and the fish dose. Therefore aqueous exposure per Kg of tissue would be similar to the highest body burden exposure time point following injection. Specifically, if the injection dose (single exposure) were 5 mg/Kg, then an equivalent aqueous exposure dose would be 5 mg/L. However, the data demonstrates that a constant exposure of 0.0002 mg/L induced more Vtg mRNA than a single exposure of 5 mg/Kg.

The difference between routes of exposure probably is that injected E_2 is quickly metabolized by the liver and cleared from the plasma, therefore only a fraction of the acute dose would actually be available to the target organ for the observed effects. This is supported by E_2-injection studies in fathead minnow (*Pimephales promelas*) where they calculated 70% clearance within 6 h (Korte et al. 2000). Thus, while a large bolus of E_2 might stimulate the estrogen receptor initially resulting in the induction of Vtg mRNA transcription, it would not be expected to persist since most of it is cleared by 6 h. Therefore, the equilibrium set up between bound ligand/receptor and the free ligand/receptor would actually favor the unbound species as E_2 is rapidly metabolized, resulting in low ER-mediated gene transcription. This amount of injected E_2 that actually reaches the estrogen receptor also depends on fraction bound to plasma proteins such as albumin or sex hormone binding globulins (Nagel et al. 1998). Therefore, understanding the route of exposure and how it relates to the observed effects is critical regarding dose selection and the interpretation of the biological end point investigated.

Aqueous Dose Response Exposures

For constant aqueous exposure to the potent estrogens, the higher doses were chosen to mimic endogenous levels of circulating estrogen in spawning females and the low doses were chosen based on the highest of levels found in the environment. Specifically, both estradiol and ethinylestradiol are commonly found in sewage effluent (Desbrow et al. 1998, Tabak et al. 1981) and are thought to represent the majority of estrogenic activity in those effluents (Desbrow et al. 1998, Purdom et al. 1994). Concentrations up to 0.8 ng/L of ethinylestradiol and up to 3.7 ng/L estradiol have been reported in water samples from wastewater treatment plants in the United States (Snyder

et al. 1999), while in the United Kingdom, E_2 and EE_2 in sewage effluent have been measured up to 40 and 7 ng/L respectively (Desbrow et al. 1998). Pilot data indicated that DES in SHM was more potent than E_2 and EE_2, therefore two lower doses were examined in addition to three comparative doses.

The observed thresholds for Vtg mRNA and protein accumulation were nominal exposure concentrations of 20 ng DES/L, 100 ng EE_2/L, and 200 ng E_2/L (Figures 4-8 and 4-9), actual measured water concentrations for E_2 and EE_2 are listed in Table 4-1. Because of the large concentration gap among doses, the actual threshold exposure concentrations are probably lower than observed. For example, 100 ng/L estradiol induced a strong Vtg mRNA response when used later as a control during the xenoestrogen exposures (Figure 4-12A). The threshold concentration for E_2 (100 ng/L) is the same as that reported in fathead minnow (Panter et al. 1998). Binding affinity for the fish estrogen receptor would predict that DES is less biologically potent than both E_2 and EE_2 (Nimrod & Benson 1997). This is just one example of how *in vitro* assays do not accurately predict *in vivo* responses (assuming that the actual DES concentration in the SHM experiments is consistent with the measured amounts of E_2 and EE_2). In this case, there is evidence that serum or sex hormone-binding globulins reduce the ability of E_2 and EE_2, but not DES, to activate the estrogen receptor (Arnold et al. 1996). Evidence for effects of serum protein binding has been shown in fish as well (Milligan et al. 1998). The data support the possibility that some structurally atypical estrogens may be biologically active at lower concentrations than structural estradiol mimics (such as EE_2).

For all exposures of E_2, DES, and EE_2 over 100 ng/L, induction of Vtg mRNA and protein was observed. Up regulation of Vtg mRNA by EE_2 exhibited a clear dose

dependent relationship for the duration of the experiment. Diethylstilbestrol induction of Vtg mRNA accumulation was also dose dependent. Unexpectantly, by 10 days of DES exposure Vtg mRNA levels decreased 3.4- and 2-fold at the lower doses (20 and 100 ng/L respectively), and increased at the highest dose (200 ng/L). This dose effect was not observed in the protein values, which increased over time. It is unclear at this time what mechanism may be responsible for this apparent inverted-U shaped respose of the mRNA at the lower doses.

In the case of E_2, there was a disparate Vtg mRNA induction relationship with dose. By one day of exposure all fish appeared to be synthesizing maximal levels of mRNA, yet the amount of Vtg protein accumulated appeared to follow a dose response. The inconsistency of this dose response relationship was not expected and currently isn't understood. None of the Vtg mRNA data shown in Figure 4-8, the E_2, DES, and EE_2 experiments, was corrected for β-actin. However, all the E_2 and some of the EE_2 Vtg mRNA data was corrected for β-actin but did not change the patterns of expression with dose and time (data not shown). Possible reasons for variation in Vtg mRNA transcription based on dose include: available plasma protein binding sites, concentration-dependent metabolism and degradation of ligand, receptor number and affinity, and stage of development (Campbell et al. 1994, Lazier et al. 1985, Smith & Thomas 1991).

In addition to characterizing the Vtg mRNA response to these potent estrogens, identifying additional estrogen-regulated genes was a priority. This is important because not many estrogen-regulated genes have been identified compared to the plethora of physiological actions of estrogen. Differential display RT-PCR is a technique that

enables the recovery and identification of differentially regulated genes (Liang & Pardee 1992). Using this technique it is possible to develop gene induction fingerprints, characteristic for specific hormone-active agents (Denslow et al. 1999a, Denslow et al. 2001). Figure 4-10 illustrates how DES and EE_2 exhibit virtually the same gene induction fingerprint as E_2. Several different primer pairs have been investigated resulting in the sequencing of several gene fragments, most of which have yet to be identified. Some of the few genes that have been positively found are vitellogenin, transferrin, ZP2 and ZP3. Vtg and both zona radiata proteins have been well documented as estrogen-inducible genes (Arukwe et al. 2000, Oppen-Berntsen et al. 1994). Estradiol induction of ZP2 in male SHM was validated by Northern blot (Figure 4-11). Preliminary results indicate that SHM transferrin is down regulated by E_2 in males (Figure 4-16). Estrogen response elements have been found in promoter regions of fish transferrin (Mikawa et al. 1996), but in mice it was reported to be upregulated by E_2 (Teng, 1995, Teng, 1999).

The doses chosen for constant aqueous exposure to the potential xenoestrogens were roughly one or two orders of magnitude greater than for the potent estrogens. The concentrations chosen for NP and the lower range of ES doses were based on previously measured values documented in sewage effluent, benthic sediment, and natural receiving waters. (Blackburn et al. 1999, Miles & Pfeuffer 1997, Sekela et al. 1999) MXC doses studied were approximately 50% serial dilutions of the maximal acceptable toxicant concentration (23 μg/L) for SHM (U.S.Environmental Protection Agency 1977).

Nonylphenol and methoxychlor induced Vtg mRNA with a similar overall rate of accumulation over time, but less potent than the natural and synthetic estrogens. The

estrogenic responses correlate with those predicted by the *in vitro* and *in vivo* assays described earlier for these chemicals. The only exception was that binding assays did not predict the estrogenicity of MXC. The bioactivation necessary for MXC estrogenicity has been described previously (Bulger et al. 1978, Schlenk et al. 1998).

The threshold observed for Vtg mRNA activation was nominal concentrations of 10 μg/L NP and 1.5 μg/L MXC. These lowest levels tested were just barely enough to detect plasma Vtg. This dose range is comparable to that seen in rainbow trout, where 3 days at the lowest dose, 10 μg/L NP, was enough to induce Vtg mRNA (Lech et al. 1996). Nonylphenol induced a dose-dependent increase in Vtg mRNA during days 2 and 5 (Figure 4-12), which is consistent with the qualitative up regulation of Vtg mRNA in rainbow trout induced by 1 day of exposure to 50 μg/L NP (Lech et al. 1996). Vitellogenin mRNA induction following MXC exposure exhibited a temporal and dose-related pattern. The lower the dose of MXC, the longer the delay in Vtg mRNA induction. This delay can be explained by the time necessary to convert MXC to its estrogenic mono- or bis-demethylated metabolites (Bulger et al. 1978, Schlenk et al. 1998). The temporal delay is clearly evident at the protein level, with 5-, 13-, and 35-day delays in plasma Vtg at MXC concentrations of 6, 3, and 1.5 μg/L (Figure 4-13). The SHM data indicates that MXC is a slightly more potent estrogen than NP, based on Vtg expression. This correlates well with their relative inhibitory potencies of 0.015 and 0.049% (MXC and NP respectively) in channel catfish (Nimrod & Benson 1997).

None of the tested endosulfan exposure concentrations induced Vtg mRNA or protein over controls. This is in disagreement with estrogen-dependent cell proliferation in MCF-7 cells, trout recombinant ER transcriptional activation assays, and Vtg mRNA

induction in trout hepatocytes, all of which indicate that ES is estrogenic *in vitro* (Petit et al. 1997, Soto et al. 1996). This lack of estrogenicity by ES is in agreement with other *in vitro* assays and the *in vivo* mouse uterotrophic assay (Shelby et al. 1996, Vonier et al. 1996). The data suggests that the pharmacokinetics of uptake, tissue distribution and metabolism negate the possible *in vitro* estrogenic actions of endosulfan in the whole animal. Since endosulfan is commercially available as a mixture of two endosulfan isomers, α and β (typically 65 to 70% α and 30 to 35%β), it is possible that other ratios of these isomers could be more estrogenic than those tested here.

Decreased Vitellogenin Levels After Exposure

The persistence of estradiol- and nonylphenol-induced Vtg mRNA after placement in clean water was investigated to determine how long this biomarker persisted following constant exposure. The decrease in vitellogenin mRNA levels in estradiol-exposed groups was slow at 2 days, followed by a rapid drop to control levels by 8 days in clean water (Figure 4-14A). This pattern of *in vivo* depuration of Vtg mRNA is virtually identical to that observed following 35 days of aqueous exposure to 50 ng EE_2/L in fathead minnow (Schmid et al. 2001). Decreased Vtg mRNA levels in NP-exposed groups dropped immediately by 2 days in clean water, but had still not returned to basal levels by 8 days (Figure 4-14B). These data suggests a biphasic removal mechanism of the steady state Vtg mRNA levels immediately following transfer to clean water. After the first two days in clean water it is apparent that the mechanisms behind decreasing Vtg mRNA levels are distinct between E_2 and NP. In addition to estrogen-induced mRNA stability; plasma protein binding, levels of activated receptor, and the recruitment of the

necessary nuclear proteins could all play a possible role in the differences seen between Vtg mRNA decreases following E_2 or NP exposure.

The decay rate of estradiol-induced Vtg mRNA has been studied in cell culture by artificially interrupting transcription with actinomycin-D. These *in vitro* studies demonstrated a 10 or 16 h half-life of Vtg mRNA in rainbow trout and frog respectively (Brock & Shapiro 1983, Flouriot et al. 1996). Decreased Vtg mRNA levels measured *in vivo* for SHM represent the gradual shift in the equilibrium from *de novo* synthesis to increased mRNA degradation; and therefore they are significantly slower. The rate of Vtg mRNA decrease over time following exposure is consistent with rates described in fathead minnow (returned to basal by 7 days) following EE_2 exposure (Schmid et al. 2001). In the presence of E_2 Vtg mRNA is stabilized, presumably by a protein also under the control of E_2 (Dodson & Shapiro 1994). In the presence of a protein synthesis inhibitor stability of the mRNA is not observed (Blume & Shapiro 1989, Flouriot et al. 1996), therefore when estradiol-exposure is withdrawn, the equilibrium shifts towards mRNA degradation. The actual decay rates for NP-induced Vtg mRNA have not been studied yet, but this information would need to account for the *in vivo* bioaccumulation and release of NP from tissue. The rapid rate of Vtg mRNA decline following aqueous NP exposure is supported by a qualitative study in rainbow trout investigating Vtg mRNA degradation following 3 days of aqueous exposure to NP (Lech et al. 1996). Using qualitative RT-PCR they reported a rapid decrease in Vtg mRNA levels by 2 and 3 days in clean water, but did not evaluate later time points.

In all experiments, treatments with E_2, DES, EE_2, NP, and MXC caused a rapid and significant accumulation of Vtg mRNA in the liver of male SHM. Although not

apparent by Northern blot, very small amounts of Vtg mRNA were detected by slot blot (approximately 0.04 ng/μg total RNA) in untreated control fish, suggesting that Vtg may be normally expressed at low levels in male fish. In tilapia, a basal level of Vtg mRNA in untreated male fish was reported as 0.05 ng/μg total RNA (Lim et al. 1991). Although no plasma Vtg was detected in control fish, the small amount of Vtg mRNA detected is probably translated into protein, but at levels below the detection limit of the assay (2 μg/mL). These data are supported by reported Vtg protein levels up to 100 ng/mL in untreated control male rainbow trout (Jobling et al. 1998).

In conclusion, these studies characterizing vitellogenin gene induction in male sheepshead minnow correlate well with previously reported data, supporting the aforementioned hypotheses. This included the time courses of Vtg mRNA and protein expression following single or double acute exposures, as well as the dose and time response patterns to constant aqueous exposures to estradiol and estrogen mimics (Bowman et al. 2000, Folmar et al. 2000, Hemmer et al. 2001). Using differential display it was possible to document the similar gene induction fingerprints among E_2, DES, and EE_2 (Denslow et al. 2001). This pattern of gene induction, in addition to discovering novel targets of gene regulation, will open the door to a better understanding of xenoestrogen regulation at the molecular level. The discovery of two Vtg genes in SHM was only the second reported in the literature for fish (Bowman et al. 2000). The greater induction of Vtg mRNA and protein accumulation with constant aqueous exposure compared to seemingly more potent acute exposure was an unexpected, but important observation (Bowman et al. 2000). The curious differences between the E_2- and NP-induced Vtg mRNA decreasing levels in clean water indicate an additional mechanistic

disparity between the effects of these two chemicals at the molecular level. Future studies with this model will lead to a better understanding of these observations and lead to a more complete picture of the gene regulation by estradiol and estrogen mimics.

Table 4-1. Nominal and actual measured water concentrations of chemicals evaluated in sheepshead minnow..

Chemical	Nominal Exposure Concentration ng/L	Actual Measured Exposure Concentration ng/L
Estradiol	20	33 (24)
	200	212 (39)
	500	699 (73)
	1000	1073 (230)
	2000	1622 (330)
Ethinylestradiol	20	24 (5)
	100	109 (29)
	200	192 (43)
	500	416 (57)
	1000	832 (259)

	Concentration µg/L	Concentration µg/L
Estradiol	0.1	65.14 (15.80)
Nonylphenol	1.0	0.64 (0.06)
	10.0	5.38 (0.45)
	20.0	11.81 (1.09)
	40.0	23.27 (3.61)
	80.0	42.67 (5.10)
Methoxychlor	1.5	1.08 (0.25)
	3.0	2.49 (0.57)
	6.0	5.59 (0.63)
	12.0	12.08 (1.95)
	24.0	18.39 (2.25)
Endosulfan	300.0	277.17 (74.79)
	600.0	403.50 (113.43)
	900.0	590.30 (150.40)
	1200.0	788.33 (212.60)

Actual exposures are the mean measured concentrations (standard deviation)
No analytical measurements were made on diethylstilbestrol levels

Table 4-2. Vitellogenin mRNA and protein induction after seven days aqueous exposure.

Chemical	Nominal Exposure Concentration ng/L	Vtg mRNA ng/μg	Vtg protein mg/mL
Control	0	0.04 (0.001)	0.00 (0.00)
E_2	200	2.84 (1.310)	18.44 (7.53)
E_2	2000	119.00 (36.960) *	63.65 (11.06) *
EE_2	100	1.25 (0.580)	15.46 (5.07) **
EE_2	1000	16.3 (4.250) *	57.39 (9.88) *

Data is presented as the mean (+/- standard deviation)
*P<0.05, **P=0.0554

Figure 4-1. Sheepshead minnow (*Cyprinidon variegatus*). Upper fish is female, typically lighter in color and smaller. Lower fish is male, normally larger and darker, especially on tips of fins.

Figure 4-2. RT-PCR on estrogen-treated sheepshead minnow RNA. Lane 1, RNA from an E_2-injected (>5 mg/Kg) fish and lane 2 is an untreated male SHM. Lane 3 & 4 are RNA samples from fish exposed in the water to 200 and 2000 ng/L E_2, respectively. Lanes 5 & 6 are RNA samples from fish exposed to 100 and 1000 ng/L EE_2 in water. Lane 7 is the RT control (no RNA), lane 8 is a PCR positive control (SHM Vtg plasmid DNA), and lane 9 is the PCR negative control (no RT reaction template). Size markers are as follows from top to bottom: 1000, 750, 500, 300, and 150 bp.

```
SHMVTG2  TSEIKFIVLL KRDQTTERNE ISVKIENIDV DMHPKNNTIV VKVNGVEIPL
FUNVTG2  TSEIKFIVLL KRDQTAERNE ISIKIENIDV DMYPKDNAVV VKVNGVEIPL
SHMVTG1  TNELKFIVLL RKD.SSEQHH INVKISEIDI DLYPKDNNVT VKVNEMEIPH
FUNVTG1  TDELKFMVLL RKD.SSEQHH INVKISEIDI DMFPKDDNVT VKVNEMEIPP

SHMVTG2  NKLPYQHPTG SIQIRVREEG VSLHAPNHGL QEVFLSLNKV QVKVVDWMRG
FUNVTG2  TNLPYQHPTG NIQIRQREEG ISLHAPSHGL QEVFLSLNKV QVKVVDWMRG
SHMVTG1  SNLPYRHPTG SIEIRQSGQG IAVYAPSHGL QEVYFDRKTW KIKVADWMKG
FUNVTG1  PACLTATQQL PLKIKTKRRG LAVYAPSHGL QEVYFDRKTW RIKVADWMKG

SHMVTG2  QTCGLCGKAD GEVRQEYSTP NERVSRNATS FAHSWVLPAK S
FUNVTG2  QTCGLCGKAD GEVRQEYSTP NERVSRNATS FAHSWVLPAK S
SHMVTG1  KTCGLCGKGD GEIRQEYRTP NGRVAKNSVS FAHSWILPAE S
FUNVTG1  KTCGLCGKAD GEIRQEYHTP NGRVAKNSIS FAHSWILPAE S
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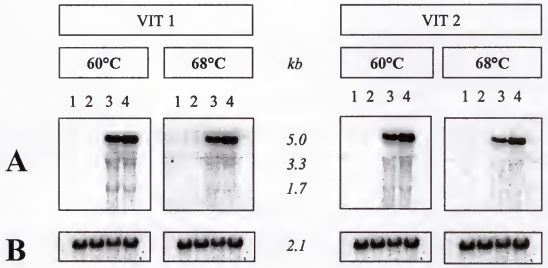
Figure 4-3. Multiple sequence alignment of sheepshead minnow VIT 1 and VIT 2. Predicted amino acid sequence for Vtgs from PCR amplified sequence. VIT 1 and 2 from SHM are lined up with respective genes from *Fundulus heteroclitus*.



C

VIT 1			VIT 2	
60°C	68°C	*kb*	60°C	68°C
62.1	60.9	*5*	76	77.9
16.3	21.9	*3.3*	13.35	12.3
21.6	17.2	*1.7*	10.63	9.8

% of total response

Figure 4-4. Northern blot analysis of the two sheepshead minnow vitellogenin mRNAs. A) A representative Northern blot of male SHM probed with SHM VIT 1 or VIT 2 cDNAs, performed under two stringency conditions (60 and 68°C) for each hybridization. Lanes 1 & 2 are RNA from control fish. Lanes 3 & 4 are RNA from E_2-injected fish; B) Same Northern blot as A, but stripped and re-probed with SHM β-actin cDNA probe; C) Average percent of total Vtg mRNA for each band on E_2-treated samples using both Vtg probes under both temperature conditions, as determined by phosphorimaging.

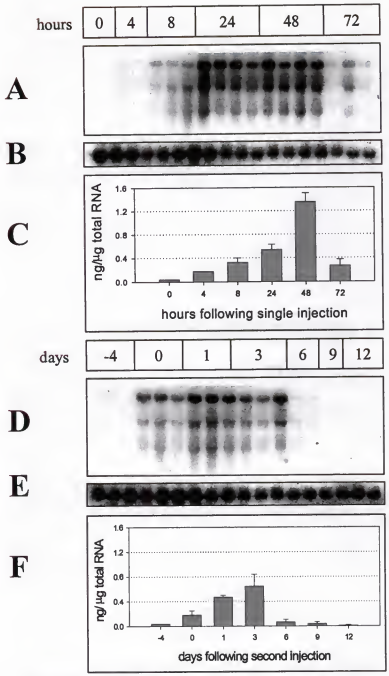


Figure 4-5. Time course of vitellogenin mRNA induction in sheepshead minnow following acute estradiol exposure. A) Vtg mRNA following single E_2-injection Northern blot; B) β-actin mRNA following single E_2 injection; C) Vtg mRNA quantification following single E_2 injection performed using slot blot analysis and a Vtg cRNA standard curve, data corrected to β-actin; D) Vtg mRNA following double E_2 injection-Northern blot; E) β-actin mRNA following double E_2 injection; F) Vtg mRNA quantification following double E_2 injection performed using slot blot analysis and a Vtg cRNA standard curve, data corrected to β-actin.

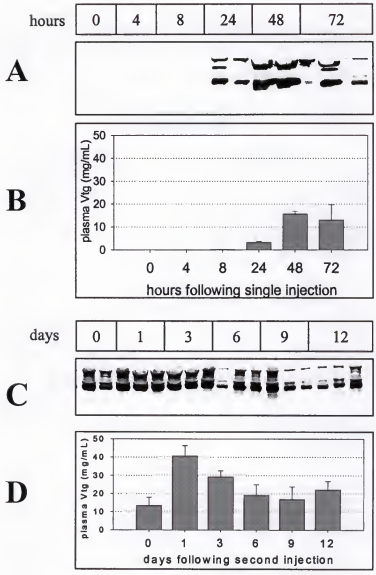


Figure 4-6. Time course of plasma vitellogenin induction in sheepshead minnow following acute estradiol exposure. A) Vtg following single E_2-injection-Western blot; B) Vtg quantification following single E_2-injection performed by ELISA; C) Vtg following double E_2 injection-Western blot; D) Vtg quantification following double E_2 injection performed by ELISA.

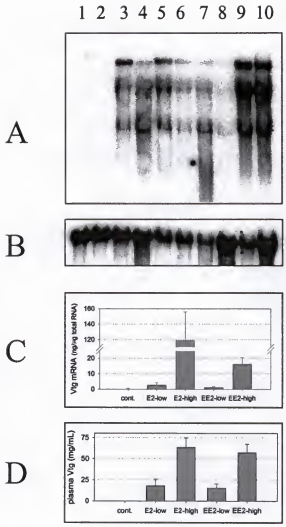


Figure 4-7. Induction of sheepshead vitellogenin mRNA and protein after seven days constant exposure. A) Northern blot of Vtg mRNA induction of fish exposed to high and low concentrations of E_2 and EE_2. Lanes 1 & 2, control males, lanes 3 & 4, E_2 (100 ng/L), lanes 5 & 6, E_2 (1000 ng/L), lanes 7 & 8, EE_2 (200 ng/ L), lanes 9 & 10, EE_2 (2000 ng/L); B) Same Northern blot as A, but β-actin mRNA; C) Vtg mRNA quantification performed using slot blot analysis and a Vtg cRNA standard curve; D) Vtg plasma protein quantification performed by ELISA.

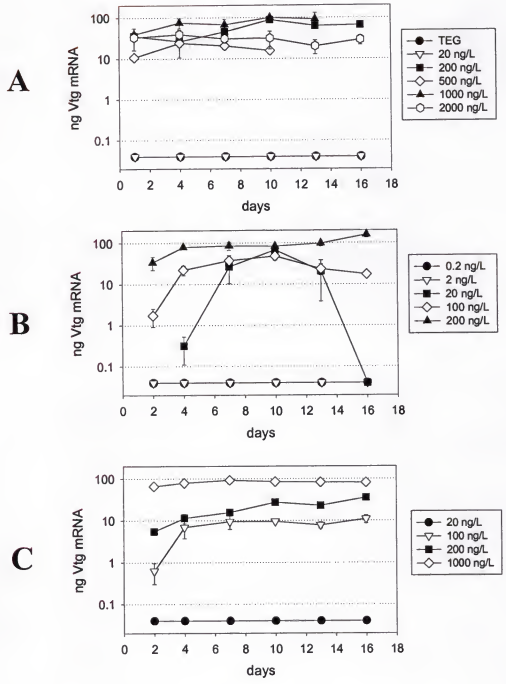


Figure 4-8. Induction of sheepshead minnow vitellogenin mRNA following constant aqueous exposure. A) Estradiol; B) Diethylstilbestrol; C) Ethinylestradiol.

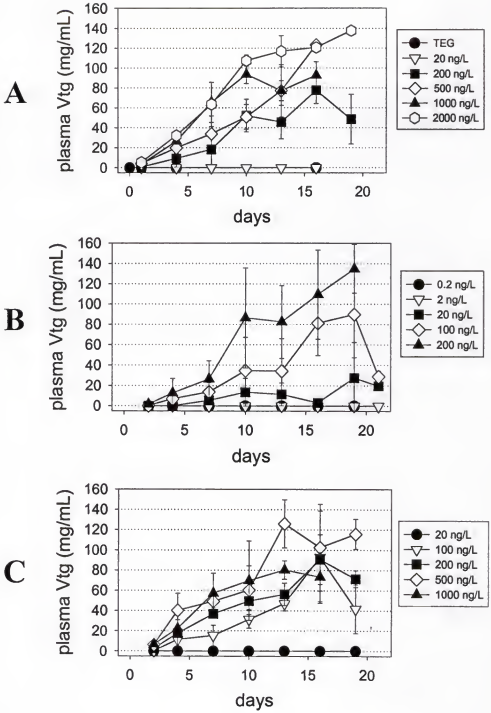


Figure 4-9. Induction of sheepshead minnow plasma vitellogenin following constant aqueous exposure. A) Estradiol; B) Diethylstilbestrol; C) Ethinylestradiol.

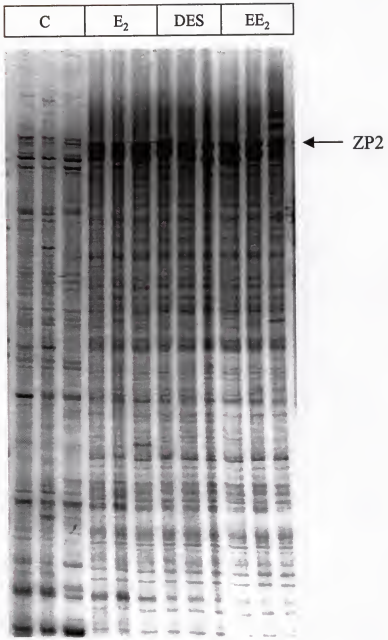


Figure 4-10. Sheepshead minnow differential display primer pair G-23. Similar mRNA regulation by all three estrogens compared to control. Arrow indicates band identified and verifed to be ZP2. Three separate fish liver RNA (lanes) analyzed per time point.

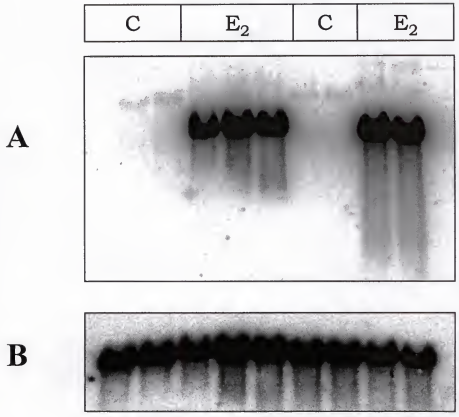


Figure 4-11. Estradiol-induced ZP2 in sheepshead minnow by Northern blot. Verification of differential display result. A) Control and E_2-induced mRNA hybridized with SHM ZP2 probe; B) Same blot showing β-actin mRNA for all samples.

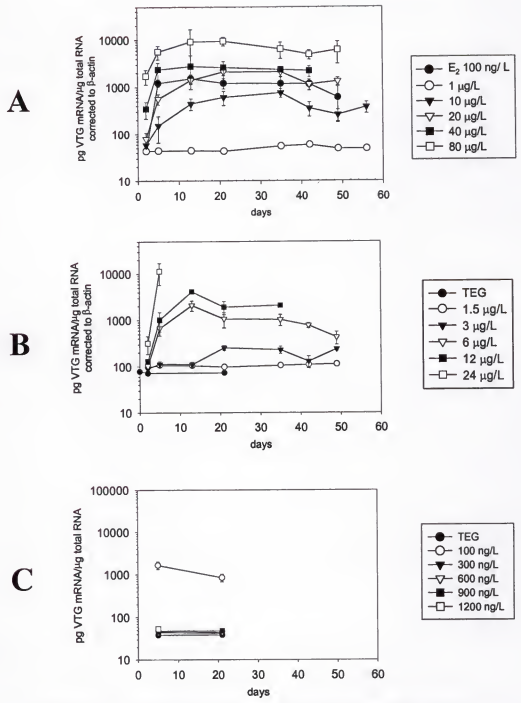


Figure 4-12. Induction of sheepshead minnow vitellogenin mRNA following constant aqueous exposure to xenoestrogens. A) Nonylphenol; B) Methoxychlor; C) Endosulfan.

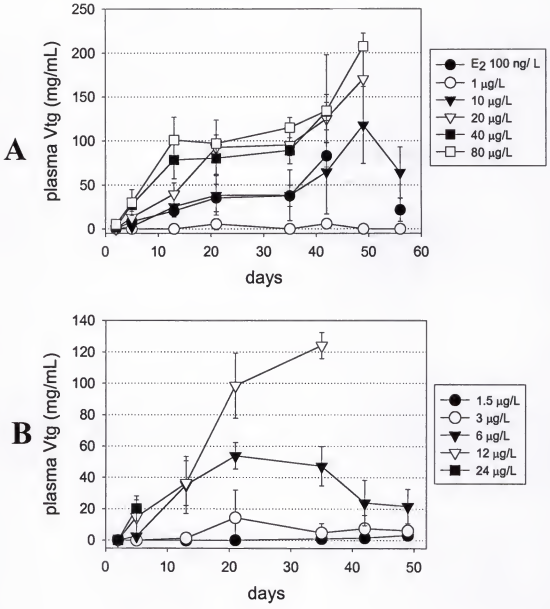


Figure 4-13. Induction of sheepshead minnow plasma vitellogenin following constant aqueous exposure to xenoestrogens. A) Nonylphenol; B) Methoxychlor.

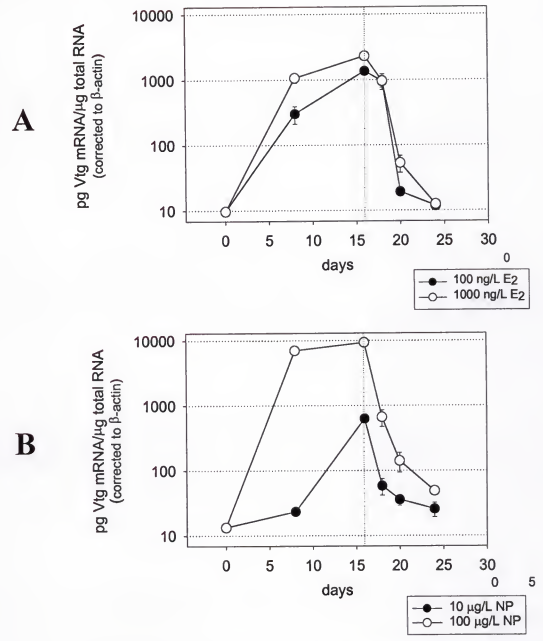


Figure 4-14. Decreased levels of sheepshead minnow vitellogenin mRNA following 16 day exposure. A) Estradioll; B) Nonylphenol.

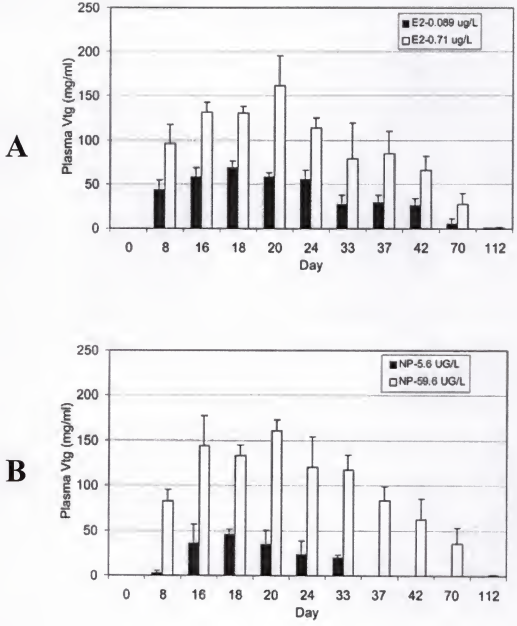


Figure 4-15. Decreased levels of sheepshead minnow plasma vitellogenin following 16 day exposure. A) Estradiol; B) Nonylphenol.

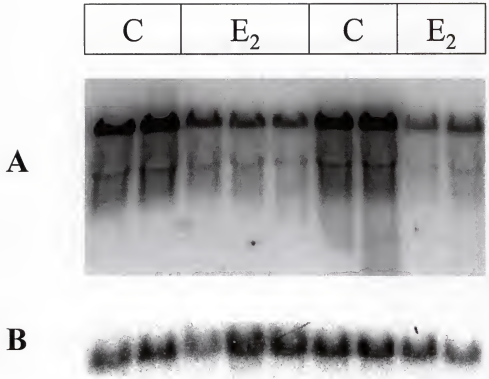


Figure 4-16. Estradiol-depressed transferrin mRNA in sheepshead minnow by Northern blot. Verification of differential display result. A) Control and E_2-induced mRNA hybridized with SHM transferrin probe; B) Same blot showing β-actin mRNA for all samples.

CHAPTER 5
CONCLUSIONS

The research conducted and presented in this dissertation used two teleost models to study the same biological pathway in distinct experimental designs specific to advantages provided by each model. One advantage of using largemouth bass (LMB), a freshwater fish, is its reproductive sensitivity to environmental perturbation. This piscivorous species also bioaccumulates xenobiotics since it is at the top of the food chain for fish. These reasons and its large geographical distribution make it a valuable sentinel species to environmental contamination. Another advantage was that reproduction in LMB is synchronized over the course of the whole year. This means that at any one time all the fish used in an experiment are at approximately the same reproductive stage. This is valuable since endogenous reproductive hormones can affect these estrogen receptor (ER)-mediated end points. Histology was done for all LMB samples to verify reproductive stage within each experiment. Studies in LMB were conducted to evaluate ER-mediated effects from environmental contaminants that behave as estrogen mimics. Specifically, the regulation of ER and vitellogenin (Vtg) mRNAs and Vtg protein were investigated following acute exposures of LMB to estradiol. An *in vitro* culture system was also developed using LMB primary hepatocytes with the purpose of providing a relevant model to study mechanisms of disruption by xenoestrogens at the molecular level.

Sheephead minnow (SHM), by comparison, is a much smaller, estuarine fish with a shorter generation time (3 to 4 months) relative to LMB (12 months). What this means

is that at any one time a population of SHM will be heterogenous in terms of its reproductive stage. Their small size also limited the amount of information we could recover from these samples, such as plasma steroids since biological sample was limited in volume. However, this model species has been studied previously and because of its small size is very amiable to large-scale experimental exposures. Sheepshead minnow were used to develop and characterize an *in vivo* bioassay for estrogenic chemicals using vitellogenin as a biomarker of exposure. This model was used to study gene expression *in vivo* using both single (injection) and constant (aqueous) exposure regimens that mimic environmental levels of exposure. Despite differences in size, reproductive cycle, and habitat, both male LMB and male SHM appear to share similar E_2-induced patterns of Vtg mRNA and protein accumulation. Use of both species has provided a more complete and comparative understanding of how E_2 and estrogen mimics regulate gene expression over time at different doses.

The estrogen receptor-mediated pathway is a primary target for the effects of environmental estrogens on wildlife. When an animal is exposed to an estrogen, the compound gets into the bloodstream where it circulates mostly bound to various binding globulins. The unbound estrogen in the blood diffuses into cells and binds soluble estrogen receptors (releasing chaperone heat shock proteins). Then this ligand-bound receptor forms homodimers and binds to specific estrogen response elements in the DNA of genes under the estrogen control. This ligand-protein-DNA complex recruits the necessary co-regulator proteins, transcription factors, and RNA polymerase. Then transcription of specific mRNAs ultimately lead to translation into proteins with target physiological functions. This *de novo* synthesis of mRNAs by estrogen is considered the

primary mechanism of estrogen action. As described in Chapter 1 this pathway is very complex, including multiple receptors, phosphorylation, crosstalk with other signal transduction pathways, and many other levels of regulation over dose and time.

There are many points in this pathway that are susceptible to disruption by suspected xenoestrogens. The vulnerable points include steroid metabolism, plasma protein binding, receptor binding, receptor conformation, receptor dimerization and DNA binding, recruitment of nuclear proteins, and much more. Most, if not all, of these alterations will manifest themselves in the cell at the level of transcriptional activation. In other words, hormone-regulated transcription and translation *in vivo* are the summarized response of all the upstream binding and signal transduction events. Therefore, by measuring the regulation of mRNA and protein synthesis over time *in vivo* it is possible to begin to understand this process. This is critical for understanding the mechanism of action for various estrogen mimics. The research presented in this dissertation has focused on characterizing ER-regulated transcription. As the best *in vivo* model of this pathway, Vtg expression was investigated in both species at the mRNA and protein level. In addition to identifying additional estrogen-regulated genes, ER mRNA was characterized in the context of Vtg activation in LMB after acute estradiol exposure.

The primary hypothesis tested in both species was that estradiol and estrogen mimics would induce a reproducible pattern of gene expression with dose and time. An additional objective tested with LMB was to resolve the apparent discrepancies in the literature regarding the up regulation of ER and Vtg mRNA after acute E_2-exposure. The coordinated induction of these genes in LMB was used to test the hypothesis that ER and Vtg mRNA induction represent a primary and delayed-primary response. As discussed in

Chapter 2 and illustrated in Figure 2-21 the induction of these mRNAs in LMB supports this paradigm. The peak of Vtg mRNA induced at 2 days after E_2-injection with both LMB and SHM (Bowman et al. 2000) is very similar to that reported in fathead minnow (Korte et al. 2000) and rainbow trout (Le Guellec et al. 1988). In LMB, ER mRNA was induced in the same time frame, but its rate of accumulation peaked earlier in time relative to Vtg after acute exposure. The paradigm of primary and delayed-primary mRNA responses is described over hours (Dean & Sanders 1996), which is consistent with the results described for LMB. An earlier study in rainbow trout reported that E_2-induced ER mRNA preceded Vtg mRNA (peak at 15 days) by a period of 1 to 2 weeks (Pakdel et al. 1991) after acute exposure. This study by Pakdel supports the notion of a primary and delayed-primary response, but over a much longer time course. However, the ER and Vtg mRNA data are not consistent with that observed in LMB or with any of the previous data in the literature regarding acute Vtg mRNA induction (peaking at 2 days after exposure). Therefore, the coordinated induction of ER and Vtg mRNA in LMB is consistent with the temporal response hypothesis, but it also reflects a more realistic time course consistent with previous Vtg mRNA data in sheepshead minnow (Bowman et al, 2000), fathead minnow (Korte et al. 2000), and a different rainbow trout study (Le Guellec et al, 1988).

To better characterize the mechanisms of gene activation by estradiol and estrogen mimics an *in vitro* primary cell culture method was developed. The assay was established using Vtg mRNA induction in LMB liver cells. This *in vitro* primary hepatocyte culture model will be useful for future studies since it represents *in vivo* cellular components while allowing for virtually unlimited experimental manipulation

(Flouriot et al. 1996). This method was intended to provide a high throughput assay to measure the dose and time response of ER-mediated events by estradiol and estrogen mimics. This culture could also be used to study the rate and stability of induced ER and Vtg mRNAs after exposure to various estrogen-like chemicals. Initially these hepatocytes were difficult to culture, but the system was optimized and characterized using general cell staining and microscopy combined with cell viability and osmolality techniques. Electron microscopy was used to validate the homogeneity of cell type and morphology. The induction of Vtg mRNA by estradiol was the end point demonstrated. While it is difficult to culture hepatocytes, the conditions have been figured out for LMB and can be used as a good model to investigate ER-mediated mechanisms of action by estradol and estrogen mimics.

Both largemouth bass and sheepshead minnow respond similarly to an acute exposure to estradiol as measured by Vtg mRNA expression. On a comparative basis, adult SHM Vtg mRNA levels peak much higher (1.35 ng/μg total RNA) than adult LMB Vtg mRNA (0.53 ng/μg total RNA), although both appear to peak at 48 h post injection. In addition to species-specific characteristics, other reasons for this almost 3-fold difference could be the dose of E_2, SHM at ~5 mg/Kg and LMB at 2 mg/Kg. Another possible reason is the vehicle chosen for E_2 administration, triethylene glycol (SHM) or dimethylsulfoxide (LMB).

In addition to characterizing the acute response of estradiol on Vtg in male SHM, the effect of constant aqueous exposures were also tested. This route of exposure is much more environmentally relevant for fish. Evaluation of this more realistic exposure route was experimentally feasible in SHM, whereas similar studies in LMB would have been

extremely difficult. Various doses of E_2 (20 to 2000 ng/L) were tested over 16 days. There was no Vtg induction with 20 ng/L, but Vtg mRNA and protein were up regulated using 200 ng/L (Figure 4-8 and 4-9). In later studies we observed the lowest observed vitellogenic response at 100 ng/L. With these constant exposures, depending on the dose, Vtg mRNA accumulation peaked after 4 to 7 days of exposure and generally remained at that level for the duration of the exposure. If fish were placed in clean water after 16 days of E_2 exposure, Vtg mRNA was cleared within 8 days whereas Vtg protein was not cleared for about 2 months (Figures 4-14 and 4-15).

An important observation made from the SHM studies was the comparison between routes of exposure on Vtg mRNA induction. Vitellogenin mRNA and plasma Vtg levels elicited *in vivo* by constant low doses of E_2 and EE_2 (200 and 100 ng/L respectively) for seven days were similar to levels induced by a single high dose of E_2 by injection (5 mg/Kg). The higher chronic exposure doses (2000 and 1000 ng/L) however, resulted in Vtg mRNA and plasma Vtg levels far exceeding the lower aqueous doses and the E_2-injection studies (Figure 4-7). These results suggest that under constant estrogen exposure in the water all synthesis and degradation systems become saturated. The resulting plateau of Vtg mRNA thus represents an equilibrium of synthesis and degradation of accumulated mRNA, whereas after acute exposure this plateau level is probably not observed.

In addition to characterizing known targets of estrogen regulation, one of the objectives of these studies was to identify novel targets of estrogen regulation in these fish. Differential display RTPCR was the technique employed for this purpose. This method of characterizing full sets of mRNA expression patterns actually served two

purposes in these experiments. For the SHM experiments in particular, gene induction "fingerprints" were recovered from samples exposed to various estrogens. Figure 4-10 illustrates this idea using three potent estrogens, estradiol, diethylstilbestrol, and ethinylestradiol. This idea has also been shown in additional work done in the lab using nonylphenol, methoxychlor, and endosulfan samples. In this case nonylphenol and methoxychlor both showed similar fingerprint patterns as E_2. Endosulfan, however, looked more like controls (unpublished results) thus suggesting that it does not behave as an estrogen in SHM. If fingerprints were completed using androgens or other hormones, this fingerprinting idea of specific chemicals could be used to help screen hormone mimics to help broadly classify them towards their suspected mechanism of action.

Using differential display it is possible to cut out specific bands of interest (differentially regulated mRNAs) and identify them by sequencing. Several differentially regulated genes have been identified this way in both LMB and SHM. As expected, this method identified Vtg in both LMB and SHM. The only other gene identified in LMB so far is a disulfide isomerase-related protein, ERp72. This is the first report of an ERp72 in teleosts and appears to be the first reported regulation of ERp72 mRNA transactivation by E_2 (Figure 2-14). In SHM many more bands have been cloned and sequenced by the Denslow lab (Denslow et al. 2001), including two zona radiata proteins and transferrin. Zona radiata proteins (ZP2 and 3) have been shown in other teleosts as estrogen-inducible (Arukwe et al. 2000, Oppen-Berntsen et al. 1994), and fits into what is known regarding the estrogen regulation of egg maturation. By Northern blot verification (Figure 4-16), transferrin appears to be down regulated by estrogen treatment. This is the opposite of what is seen in mammals, where it is up regulated by E_2 (Teng, 1995, Teng,

1999). It is not clear why this gene seems to be regulated inversely compared to mammals, but this opposite regulation by E_2 is true for ER as well (ER is down regulated in mammals and up regulated in fish).

As described briefly above, the basic endocrinology of gene regulation by estradiol can be applied to relevant questions in environmental toxicology, such as endocrine disruption. As one of the possible primary mechanisms of disruption, estrogen receptor-mediated events are important to characterize following xenoestrogen exposure. This dissertation takes a comparative approach to this characterization. Largemouth bass were used to evaluate coordinate gene expression of ER and Vtg mRNA with complementary information on plasma steroids and Vtg protein. The dose response of plasma Vtg induction following estradiol, ethinylestradiol, nonylphenol, methoxychlor, and o,p'-DDT was also investigated. Both estradiol and ethinylestradiol resulted in a dose-dependent induction, but none of the other estrogen mimics, with injections up to 5 mg/Kg, induced plasma Vtg.

The sheepshead minnow model model was used as an *in vivo* bioassay for estrogen exposure (Folmar et al. 2000). This bioassay measured the dose and time response of Vtg mRNA and protein to various concentrations of three estrogens (estradiol, ethinylestradiol, and diethylstilbestrol) at low doses by water expsoure. Then the estrogenicity of three xenoestrogens (nonylphenol, methoxychlor, and endosulfan), again at environmentally-relevant concentrations, was tested *in vivo* (Hemmer et al. 2001). To identify the persistence of these markers in clean water following exposure, a study of Vtg mRNA and protein was done using estradiol and nonylphenol (Figure 4-14 and 4-15). This experiment shows that these chemicals result in lead to different rates of

decreasing Vtg mRNA levels following transfer to clean water, with nonylphenol-induced Vtg mRNA slower probably due to its slower release from tissue following exposure.

The discovery of two Vtg genes in SHM and LMB (unpublished results) is intriguing. The function and importance of these two genes in reproduction and as a biomarker remains to be investigated. The presence of low basal levels of Vtg mRNA and protein in male fish are also an important observation that is consistent with other reports (Jobling et al. 1998, Lim et al. 1991). These factors (multiple genes and background levels) are important considerations when trying to determine how much and when xenoestrogen exposure in the wild has occurred.

The sensitivity of Vtg mRNA and protein to different concentrations of E_2-exposure, both acute and chronic, is valuable information for using this gene as a biomarker of estrogen exposure in the wild. This is because Vtg has been used for years as a biomarker of exposure, but still very little was done until now characterizing its dose response *in vivo* at the mRNA and protein level. As many LMB and SHM experiments show, Vtg mRNA and protein are distinct biomarkers of estrogen exposure *in vivo*, and can represent different types of exposures. Vitellogenin mRNA levels reflect an immediate exposure to an estrogen, diminishing after an acute exposure is terminated. Vtg plasma levels, on the other hand, remain stable for a longer time and thus can be used for charting contamination fluxes. Also by measuring the *in vivo* increase in both Vtg mRNA and protein, it is possible to better characterize different levels of the ER-mediated pathway representing activation of transcription in the nucleus to mature protein secreted into the plasma of the animal. The method development for quantitating

Vtg mRNA and protein, as well as establishing these responses to E_2 provide the necessary baseline data by which to evaluate the effects of environmental xenoestrogens (Figure 4-12 and 4-13).

By understanding the regulation of E_2-induced mRNA and protein expression with different routes of exposure (as described previously for SHM), it may be possible to predict unknown consequences of estrogen mimics. This may explain why a 5 mg/Kg dose of nonylphenol or methoxychlor did not induce a measurable amount of plasma Vtg in LMB 48 h post injection (Table 2-1), whereas a constant exposure to these compounds (3 to 10 μg/L) did induce plasma Vtg in SHM over time (Figure 4-13).

In the United Kingdom up to 180 μg NP/L has been reported in final effluents into the environment (Blackburn & Waldock 1995). Lower levels of NP (0.11 to 15.1 μg/L) have been reported in the United States and Canada (Talmadge 1994, Kubeck & Naylor 1990, Lee & Peart 1995). Evidence is accumulating that alkylphenols such as NP and 4-*tert*-pentylphenol (TPP) are capable of inhibiting spermatogenesis in trout (Jobling et al. 1996) and carp (Gimeno et al. 1998b) at 30 and 90 μg/L respectively. 4-*tert*-pentylphenol also induces the formation of an oviduct in the undifferentiated male reproductive tract (Gimeno et al. 1996, Gimeno et al. 1998b), which was permanent following transfer to clean water (Gimeno et al. 1997). Male medaka exposed to octylphenol (OP), then mated in clean water led to decreased egg number, decreased percent fertilization, and decreased embryo survival in the resulting offspring (Gronen et al. 1999). An 86% incidence of testis-ova (an intersex condition where both testicular and ovarian tissue are present in the gonad) occurred in medaka exposed to 50 to 100 μg NP/ L from hatch to 3 months of age (Gray & Metcalfe 1996). This inhibition of

testicular growth, feminization of the reproductive tract, and high incidence of testis-ova in animals exposed to environmentally-relevant levels of alkylphenols is striking and certainly merits additional attention in the regulation of this class of xenoestrogens. Some studies have even begun to link these adverse reproductive effects with increased Vtg levels in male fish (Gronen et al. 1999, Jobling et al. 1996). If a conclusive mechanistic link could be established, the use of Vtg in males could serve as a biomarker of effect, not just as its current utility as an exposure marker. Therefore detailed characterization of Vtg with dose and time in sentinel species such as LMB and SHM may serve the ecological regulatory community.

It is important to establish basic biological parameters for some of these sentinel species in the environment. Such factors as age and route of exposure can significantly impact how these organisms may or may not be affected. Once these model systems are established, it is possible to identify anomalies following exposure to xenoestrogens. Identifying abnormal plasma E_2 levels following nonylphenol or methoxychlor injection in LMB is one example.

In conclusion, estrogen receptor and vitellogenin mRNA complemented with plasma Vtg is a robust system to characterize *in vivo* effects of estradiol and estrogen mimics in important environmental species. The investigation of baseline mRNA and protein responses in two distinct teleost species, largemouth bass and sheepshead minnow, provides some of the basic molecular endocrinology necessary for evaluating disruption of xenoestrogens in the environment or in the laboratory. In addition to *in vivo* characterization, the development of an *in vitro* primary hepatocyte model using a species of environmental concern can be used in future experiments to test specific mechanisms

of xenoestrogen action in the liver. The research presented in this dissertation is the first time estrogen actions at the mRNA level have been investigated in either SHM or LMB. Future research using these models will build on the basic endocrinology presented here.

Future work based on this body of research could address more specific roles of ER and Vtg during reproduction and development following xenoestrogen exposure. Additional validation of the LMB primary hepatocyte model previously described would provide a valuable and biologically relevant tool to tease apart specific mechanisms behind the differential gene regulation of ER and Vtg with xenoestrogen exposure. Future directions using these specific models will focus on two areas. The first is the characterization of ER isotypes in LMB and how they function during reproduction following xenoestrogen exposure. The second is investigating the overall mRNA response to contaminants using microarray technology in both LMB and SHM. With the research presented here, the application of new molecular technologies, and impacts on whole animal reproduction will hopefully lead to a better understanding of the mechanisms behind endocrine disruption by xenoestrogens.

LIST OF REFERENCES

Ahel, M. and Giger, W. (1985). Determination of alkylphenols and alkylphenol mono- and diethoxylates in environmental samples by high-performance liquid chromatography. *Anal. Chem.* **57**, 1577-1583.

Aherne, G. W. and Briggs, R. (1989). The relevance of the presence of certain synthetic steroids in the aquatic environment. *J. Pharm. Pharmacol.* **41**, 735-736.

Allan, R. J., Ball, A. J., Cairns, V. W., Fox, G. A., Gilman, A. P., Peakall, D. B., Piekarz, D. A., Van Oostdam, J. C., Villeneuve, D. C., Williams, D. T. (1991). Toxic Chemicals in the Great Lakes and Associated Effects: Volume II, Effects. Ottawa, Environment Canada, Department of Fisheries and Oceans, Health and Welfare Canada.

Allen, E. and Doisy, E. A. (1923). An ovarian hormone. Preliminary report on its localization, extraction and partial purification, and action in test animals. *J. Am. Med. Assoc.* **81**, 819-821.

Allen, E. and Doisy, E. A. (1924). The induction of a sexually mature condition in immature females by injection of the ovarian follicular hormone. *Am. J. Physiol.* **69**, 577-588.

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nuc. Acids Res.* **25**, 3389-3402.

Anderson, M. J., Olsen, H., Matsumura, F., and Hinton, D. E. (1996). In vivo modulation of 17β-estradiol-induced vitellogenin synthesis and estrogen receptor in rainbow trout (*Oncorhynchus mykiss*) liver cells by β-naphthoflavone. *Toxicol. Appl. Pharmacol.* **137**, 210-218.

Arnold, S. F., Collins, B. M., Robinson, M. K., Guillette, L. J. Jr., and McLachlan, J. A. (1996). Differential interaction of natural and synthetic estrogens with extracellular binding proteins in a yeast estrogen screen. *Steroids* **61**, 642-646.

Arnold, S. F., Obourn, J. D., Jaffe, H., and Notides, A. C. (1995). Phosphorylation of the human estrogen receptor by mitogen-activated protein kinase and casein kinase II: Consequence on DNA binding. *J. Steroid Biochem. Mol. Biol.* **55**, 163-172.

Arukwe, A., Celius, T., Walther, B. T., and Goksoyr, A. (1998). Plasma levels of vitellogenin and eggshell zona radiata proteins in 4-nonylphenol and o,p'-DDT treated juvenile Atlantic salmon (*Salmo salar*). *Mar. Environ. Res.* **46**, 133-136.

Arukwe, A., Celius, T., Walther, B. T., and Goksoyr, A. (2000). Effects of xenoestrogen treatment on zona radiata protein and vitellogenin expression in Atlantic salmon (*Salmo salar*). *Aquat. Toxicol.* **49**, 159-170.

Arukwe, A., Forlin, L., and Goksoyr, A. (1997). Xenobiotic and steroid biotransformation enzymes in Atlantic salmon (*Salmo salar*) liver treated with an estrogenic compound, 4-nonylphenol. *Environ. Toxicol. Chem.* **16**, 2576-2583.

Baker, H. J. and Shapiro, D. J. (1978). Rapid accumulation of vitellogenin messenger RNA during secondary estrogen stimulation of Xenopus laevis. *J. Biol. Chem.* **253**, 4521-4524.

Baski, S. M. and Frazier, J. M. (1990). Isolated fish hepatocytes-model systems for toxicology research. *Aquat. Toxicol.* **16**, 229-256.

Benson, D. A., Boguski, M. S., Lipman, D. J., Ostell, J., Ouellette, B. F., Rapp, B. A., and Wheeler, D. L. (1999). GenBank. *Nuc. Acids Res.* **27**, 12-17.

Bergeron, J. M., Crews, D., and McLachlan, J. A. (1994). PCBs as environmental estrogens: Turtle sex determination as a biomarker of environmental contamination. *Environ. Health Perspect.* **102**, 780-781.

Berry, M. N. and Friend, D. S. (1969). High yield preparation of isolated rat liver parenchymal cells: A biochemical and fine structural study. *J. Cell. Biol.* **43**, 506-520.

Bevans, H. E., Goodbred, S. L., Miesner, J. F., Watkins, S. A., Gross, T. S., Denslow, N. D., Schoeb, T. (1996). Synthetic organic compounds and carp endocrinology and histology in Las Vegas Wash and Las Vegas and Callville Bays of Lake Mead, Nevada, 1992 and 1995. Water Resources Investigation Report 96-4266, pp. 1-12. Denver, CO, U.S. Geological Survey.

Bitman, J. and Cecil, H. C. (1970). Estrogenic activity of DDT analogs and polychlorinated biphenyls. *J. Agric. Food Chem.* **18**, 1108-1112.

Bitman, J., Cecil, H. C., Harris, S. J., and Fries, G. F. (1968). Estrogenic activity of o,p'-DDT in the mammalian uterus and avian oviduct. *Science* **162**, 371-372.

Blackburn, M. A., Kirby, S. J., and Waldock, M. J. (1999). Concentrations of alkylphenol polyethoxylates entering UK estuaries. *Mar. Pollut. Bull.* **38**, 109-118.

Blackburn, M. A. and Waldock, M. J. (1995). Concentrations of alkylphenols in rivers and estuaries in England and Wales. *Water Res.* **29**, 1623-1629.

Blair, J. B., Miller, M. R., Pack, D., Barnes, R., Teh, S. J., and Hinton, D. E. (1990). Isolated trout liver cells: establishing short-term primary cultures exhibiting cell-to-cell interactions. *In Vitro Cell. Dev. Biol.* **26**, 237-249.

Blume, J. E. and Shapiro, D. J. (1989). Ribosome loading, but not protein synthesis, is required for estrogen stabilization of Xenopus laevis vitellogenin mRNA. *Nuc. Acids Res.* **17** , 9003-9014.

Bowman, C. J. and Denslow, N. D. (1999). Development and validation of a species- and gene-specific molecular biomarker: Vitellogenin mRNA in largemouth bass (*Micropterus salmoides*). *Ecotoxicology* **8**, 399-416.

Bowman, C. J., Kroll, K. J., Hemmer, M. J., Folmar, L. C., and Denslow, N. D. (2000). Estrogen-induced vitellogenin mRNA and protein in sheepshead minnow (*Cyprinodon variegatus*). *Gen. Comp. Endocrinol.* **120**, 300-313.

Brock, M. L. and Shapiro, D. J. (1983). Estrogen stabilizes vitellogenin mRNA against cytoplasmic degradation. *Cell* **34**, 207-214.

Brotons, J. A., Olea-Serrano, M. F., Villalobos, M., Pedraza, V., and Olea, N. (1995). Xenoestrogens released from lacquer coatings in food cans. *Environ. Health Perspect.* **103**, 608-612.

Brusle, J. and Anadon, G.G. (1996). The structure and function of fish liver. In: Fish Morphology, Horizon of New Research (J.S.D. Munshi and H.M. Dutta, Eds.), pp. 77-93. Science Publishers, Inc., New Delhi.

Bulger, W. H., Muccitelli, R. M., and Kupfer, D. (1978). Studies on the in vivo and in vitro estrogenic activities of methoxychlor and its metabolites. Role of hepatic mono-oxygenase in methoxychlor activation. *Biochem. Pharmacol.* **27**, 2417-2423.

Burch, J. B. E. and Evans, M. I. (1986). Chromatin structural transitions and the phenomenon of vitellogenin gene memory in chickens. *Mol. Cell. Biol.* **6**, 1886-1893.

Burch, J. B. E., Evans, M. I., Friedman, T. M., and O'Malley, B. W. (1988). Two functional estrogen response elements are located upstream of the major chicken vitellogenin gene. *Mol. Cell. Biol.* **8**, 1123-1131.

Campbell, P. M., Pottinger, T. G., and Sumpter, J. P. (1994). Changes in the affinity of estrogen and androgen receptors accompany changes in receptor abundance in brown and rainbow trout. *Gen. Comp. Endocrinol.* **94**, 329-340.

Carlsen, E., Giwercman, A., Keiding, N., and Skakkebaek, N. E. (1995). Declining semen quality and increasing incidence of testicular cancer: is there a common cause? *Environ. Health Perspect.* **103**, 137-139.

Carson-Jurica, M. A., Schrader, W. T., and O'Malley, B. W. (1990). Steroid receptor family: structure and functions. *Endocrin. Rev.* **11**, 201-220.

Carson, R. (1962). Silent Spring, pp. 1-368. Houghton Mifflin Company, Boston.

Casillas, E., Misitano, D., Johnson, L. L., Rhodes, L. D., Collier, T. K., Stein, J. E., McCain, B. B., and Varanasi, U. (1991). Inducibility of spawning and reproductive success of female English sole (*Parophrys vetulus*) from urban and nonurban areas of Puget Sound, Washington. *Mar. Environ. Res.* **31**, 99-122.

Chan, G. C. K., Hess, P., Meenakshi, T., Carlstedt-Duke, J., Gustafsson, J. A., and Payvar, F. (1991). Delayed secondary glucocorticoid response elements: unusual nucleotide motifs specify glucocorticoid receptor binding to transcribed regions of a 2μ-globulin DNA. *J. Biol. Chem.* **266**, 22634-22644.

Chomczynski, P. and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**, 156-159.

Christiansen, L. B., Korsgaard, B., and Bjerregaard, P. (1999). The effect of 4-nonylphenol on the synthesis of vitellogenin in the flounder, *Platichthys flesus*. *Aquat. Toxicol.* **46** , 211-219.

Colborn, T., Dumanoski, D., and Myers, J. P. (1997). Our Stolen Future, pp. 1-316. Penguin Books USA, Inc., New York.

Cooke, A. S. (1973). Shell thinning in avian eggs by environmental pollutants. *Environ. Pollut.* **4**, 85-152.

Corpet, F. (1988). Multiple sequence alignment with hierarchical clustering. *Nuc. Acids Res.* **16**, 10881-10890.

Cravedi, J. P., Paris, A., Monod, G., Devaux, A., Flouriot, G., and Valotaire, Y. (1996). Maintenance of cytochrome P450 content and phase I and phase II enzyme activities in trout hepatocytes cultured as spheroidal aggregates. *Comp. Biochem. Physiol.* **113C**, 241-246.

Crews, D., Bull, J. J., and Wibbels, T. (1991). Estrogen and sex reversal in turtles: A dose-dependent phenomenon. *Gen. Comp. Endocrinol.* **81**, 357-364.

Cummings, A. M. (1990). Toxicological mechanisms of implantation failure. *Fund. Appl. Toxicol.* **15**, 571-579.

Cummings, A. M. (1997). Methoxychlor as a model for environmental estrogens. *Crit. Rev. Toxicol.* **27**, 367-379.

Dean, D. M. and Sanders, M. M. (1996). Ten years after: reclassification of steroid-responsive genes. *Mol. Endocrinol.* **10**, 1489-1495.

Denslow, N. D., Bowman, C. J., Ferguson, R. J., Lee, H. S., Hemmer, M. J., Folmar, L. C. (2001). Induction of gene expression in sheepshead minnows (*Cyprinidon variegatus*) treated with 17-β-estradiol, diethystilbestrol, or ethinylestradiol: The use of mRNA fingerprints to find inducible genes. *Gen. Comp. Endocrinol.* **121**, 250-260.

Denslow, N.D., Bowman, C.J., Robinson, G., Lee, H.S., Ferguson, R.J., Hemmer, M.J., and Folmar, L.C. (1999a). Biomarkers of endocrine disruption at the mRNA level. In: Environmental Toxicology and Risk Assessment: ASTM STP 1364 (D. Henshel, Ed.), pp. 24-35. American Society for Testing and Materials, West Conshohocken, PA.

Denslow, N.D., Chow, M., Chow, M.M., Bonomelli, S., Folmar, L.C., Heppell, S.A., and Sullivan, C.V. (1997a). Development of biomarkers for environmental contaminants affecting fish. In: Chemically Induced Alterations in Functional Development and Reproduction in Fishes (R.M. Rolland, M. Gilbertson, and R.E. Peterson, Eds.), pp. 73-86. SETAC Press, Pensacola, FL

Denslow, N. D., Chow, M. C., Kroll, K. J., and Green, L. (1999b). Vitellogenin as a biomarker of exposure for estrogen or estrogen mimics. *Ecotoxicology* **8**, 385-398.

Denslow, N.D., Chow, M.M., Folmar, L.C., Bonomelli, S.L., Heppell, S.A., and Sullivan, C.V. (1997b). Development of antibodies to teleost vitellogenins: potential biomarkers for environmental estrogens. In: Environmental Toxicology and Risk Assessment: Biomarkers and Risk Assessment: ASTM STP 1306 (D.A. Bengston and D.S. Henshel, Eds.), pp. 23-36. American Society for Testing and Materials, Philadelphia, PA.

Desbrow, C., Routledge, E. J., Brighty, G. C., Sumpter, J. P., and Waldock, M. (1998). Identification of estrogenic chemicals in STW effluent. 1. chemical fractionation and in vitro biological screening. *Environ. Sci. Technol.* **32**, 1549-1558.

Dieckmann, W. J., Davis, M. E., Rynkiewicz, L. M., and Pottinger, R. E. (1953). Does the administration of diethystilbestrol during pregnancy have therapeutic value? *Am. J. Obstet. Gynecol.* **66**, 1062-1081.

Dodson, R. E. and Shapiro, D. J. (1994). An estrogen-inducible protein binds specifically to a sequence in the 3' untranslated region of estrogen-stabilized vitellogenin mRNA. *Mol. Cell. Biol.* **14**, 3130-3138.

Dodson, R. E. and Shapiro, D. J. (1997). Vigilin, a ubiquitous protein with 14 K homology domains, is the estrogen-inducible vitellogenin mRNA 3'-untranslated region-binding protein. *J. Biol. Chem.* **272**, 12249-12252.

Dorner, A. J., Wasley, L. C., Raney, P., Haugejorden, S., Green, M., and Kaufman, R. J. (1990). The stress response in Chinese hamster ovary cells. Regulation of ERp72 and protein disulfide isomerase expression and secretion. *J. Biol. Chem.* **265**, 22029-22034.

Ecobichon, D. J. and MacKenzie, D. O. (1974). The uterotropic activity of commercial and isomerically-pure chlorobiphenyls in the rat. *Res. Commun. Chem. Pathol. Pharmacol.* **9**, 85-95.

Edgren, R. A. (1994). Early oral contraceptive history: Norethynodrel is not a prohormone. *Steroids* **59**, 58-59.

Edgren, R. A., Peterson, D. L., Jones, R. C., Nagra, C. L., Smith, H., and Hughes, G. A. (1966). Biological effects of synthetic gonanes. *Rec. Prog. Horm. Res.* **22**, 305-349.

Evans, R. M. (1988). The steroid and thyroid hormone receptor superfamily. *Science* **240**, 889-895.

Ferri, S. and Sesso, A. (1981). Ultrastructural study of the endothelial cells in teleost liver sinusoids under normal and experimental conditions. *Cell Tissue Res.* **219**, 649-657.

Flouriot, G., Pakdel, F., Ducouret, B., Ledrean, Y., and Valotaire, Y. (1997). Differential regulation of two genes implicated in fish reproduction: vitellogenin and estrogen receptor genes. *Mol. Reprod. Dev.* **48**, 317-323.

Flouriot, G., Pakdel, F., Ducouret, B., and Valotaire, Y. (1995). Influence of xenobiotics on rainbow trout liver estrogen receptor and vitellogenin gene expression. *J. Mol. Endocrinol.* **15**, 143-151.

Flouriot, G., Pakdel, F., and Valotaire, Y. (1996). Transcriptional and post-transcriptional regulation of rainbow trout estrogen receptor and vitellogenin gene expression. *Mol. Cell. Endocrinol.* **124**, 173-183.

Flouriot, G., Vaillant, C., Salbert, G., Pelissero, C., Guiraud, J. M., and Valotaire, Y. (1993). Monolayer and aggregate cultures of rainbow trout hepatocytes: long-term and stable liver-specific expression in aggregates. *J. Cell Sci.* **105**, 407-416.

Folman, Y. and Pope, G. S. (1966). The interaction in the immature mouse of potent oestrogens with coumestrol, genistein and other utero-vagino-trophic compounds of low potency. *J. Endocrinol.* **34**, 215-225.

Folmar, L. C., Denslow, N. D., Rao, V., Chow, M., Crain, D. A., Enbolm, J., Marcino, J., and Guillette, L. J. Jr. (1996). Vitellogenin induction and reduced serum testosterone concentrations in feral male carp (*Cyprinus carpio*) captured near a major metropolitan sewage treatment plant. *Environ. Health Perspect.* **104**, 1096-1101.

Folmar, L. C., Gardner, G. R., Schreibman, M. P., Magliulo-Cepriano, L., Mills, L. J., Zaroogian, G., Gutjahr-Gobell, R., Haebler, R., Horowitz, D. B., and Denslow, N. D. (2001). Vitellogenin-induced pathology in male summer flounder (*Paralichthys dentatus*). *Aquat. Toxicol.* **51**, 431-441.

Folmar, L. C., Hemmer, M., Hemmer, R., Bowman, C., Kroll, K., and Denslow, N. D. (2000). Comparative estrogenicity of estradiol, ethynyl estradiol and diethylstilbestrol in an in vivo, male sheepshead minnow (*Cyprinidon variegatus*), vitellogenin bioassay. *Aquat. Toxicol.* **49**, 77-88.

Fox, G. A., Weseloh, D. V., Kubiak, T. J., and Erdman, T. C. (1991). Reproductive outcomes in colonial fish-eating birds: A biomarker for developmental toxicants in Great Lakes food chains. *J. Great Lakes Res.* **17**, 153-157.

Freedman, R. B. (1984). Native disulphide bond formation in protein biosynthesis: evidence for the role of protein disulphide isomerase. *TIBS* **10**, 438-441.

Freedman, R. B. (1989). Protein disulfide isomerase: multiple roles in the modification of nascent secretory proteins. *Cell* **57**, 1069-1072.

Freshney, R.I. (2000). Primary culture. In: Culture of Animal Cells: A Manual of Basic Technique pp. 149-176. Wiley Liss, New York.

Fritsch, M., Leary, C. M., Furlow, J. D., Ahrens, H., Schuh, T. J., Mueller, G. C., and Gorski, J. (1992). A ligand-induced conformational change in the estrogen receptor is localized in the steroid binding domain. *Biochemistry* **31**, 5303-5311.

Funkenstein, B., Bowman, C. J., Denslow, N. D., Cardinali, M., and Carnevali, O. (2000). Contrasting effects of estrogen on transthyretin and vitellogenin expression in males of the marine fish, *Sparus aurata*. *Mol. Cell. Endocrinol.* **167**, 33-41.

Gellert, R. J., Heinrichs, W. L., and Swerdloff, R. (1974). Effects of neonatally-administered DDT homologs on reproductive function in male and female rats. *Neuroendocrinology* **16**, 84-94.

Gerber-Huber, S., Nardelli, D., Haefliger, J. A., Cooper, D. N., Givel, F., Germond, J. E., Engel, J., Green, N. M., and Wahli, W. (1987). Precursor-product relationship between vitellogenin and the yolk proteins as derived from the complete sequence of a Xenopus vitellogenin gene. *Nuc. Acids Res.* **15**, 4737-4760.

Giesy, J. P., Ludwig, J. P., and Tillitt, D. E. (1994). Deformaties in birds of the Great Lakes region: Assigning causality. *Environ. Sci. Technol.* **28**, 128A-135A.

Giesy, J. P., Pierens, S. L., Snyder, E. M., Miles-Richardson, S. R., Kramer, V. J., Snyder, S. A., Nichols, K. M., and Villeneuve, D. A. (2000). Effects of 4-nonylphenol on fecundity and biomarkers of estrogenicity in fathead minnows (*Pimephales promelas*). *Environ. Toxicol. Chem.* **19**, 1368-1377.

Giguere, V., Yang, N., Segui, P., and Evans, R. M. (1988). Identification of a new class of steroid hormone receptors. *Nature* **331**, 91-93.

Gimeno, S., Gerritsen, A., Bowmer, T., and Komen, H. (1996). Feminization of male carp. *Nature* **384**, 221-222.

Gimeno, S., Komen, H., Gerritsen, A. G. M., and Bowmer, T. (1998b). Feminisation of young males of the common carp, *Cyprinus carpio*, exposed to 4-*tert*-pentylphenol during sexual differentiation. *Aquat. Toxicol.* **43**, 77-92.

Gimeno, S., Komen, H., Jobling, S., Sumpter, J. P., and Bowmer, T. (1998a). Demasculinisation of sexually mature male common carp, *Cyprinus carpio*, exposed to 4-*tert*-pentylphenol during spermatogenesis. *Aquat. Toxicol.* **43**, 93-109.

Gimeno, S., Komen, H., Venderbosch, P., and Bowmer, T. (1997). Disruption of sexual differentiation in genetic male common carp (*Cyprinus carpio*) exposed to an alkylphenol during different life stages. *Environ. Sci. Technol.* **31**, 2884-2890.

Glass, C. K. (1994). Differential recognition of target genes by nuclear receptor monomers, dimers, and heterodimers. *Endocrin. Rev.* **15**, 391-407.

Gray, M. A., and Metcalfe, C. D. (1996). Induction of testis-ova in Japanese medaka (*Oryzias latipes*) exposed to p-nonylphenol. *Environ. Toxicol. Chem.* **16**, 1082-1086.

Greene, G. L., Gilna, P., Waterfield, M., Baker, A., Hort, Y., and Shine, J. (1986). Sequence and expression of human estrogen receptor complementary cDNA. *Science* **231**, 1150-1154.

Gronen, S., Denslow, N., Manning, S., Barnes, S., Barnes, D., and Brouwer, M. (1999). Serum vitellogenin levels and reproductive impairment of male Japanese medaka

(*Oryzias latipes*) exposed to 4-tert-octylphenol. *Environ. Health Perspect.* **107**, 385-390.

Guillette, L. J. Jr., Gross, T. S., Masson, G. R., Matter, J. M., Percival, H. F., and Woodward, A. R. (1994). Developmental abnormalities of the gonad and abnormal sex hormone concentrations in juvenile alligators from contaminated and control lakes in Florida. *Environ. Health Perspect.* **102**, 680-688.

Guiney, P. D., Cook, P. M., Casselman, J. M., Fitzsimmons, J. D., Simonin, H. A., Zabel, E. W., and Peterson, R. E. (1996). Assessment of 2,3,7,8-tetrachlorodibenzo-p-dioxin-induced sac fry mortality in lake trout (*Salvelinus namaycush*) from different regions of the Great Lakes. *Can. J. Fish Aquat. Sci.* **53**, 2080-2092.

Hampton, J. A., McCuskey, P. A., McCuskey, R. S., and Hinton, D. E. (1985). Functional units in rainbow trout (*Salmo gairdneri*) liver: I. arrangement and histochemical properties of hepatocytes. *Anat. Rec.* **213**, 166-175.

Harries, J. E., Janbakhsh, A., Jobling, S., Matthiessen, P., Sumpter, J. P., and Tyler, C. R. (1999). Estrogenic potency of effluent from two sewage treatment works in the united kingdom. *Environ. Toxicol. Chem.* **18**, 932-937.

Harries, J. E., Sheahan, D. A., Jobling, S., Matthiessen, P., Neall, P., Routledge, E. J., Rycroft, R., Sumpter, J. P., and Tylor, T. (1996). A survey of estrogenic activity in United Kingdom inland waters. *Environ. Toxicol. Chem.* **15**, 1993-2002.

Harries, J. E., Sheahan, D. A., Jobling, S., Matthiessen, P., Neall, P., Sumpter, J. P., Tylor, T., and Zaman, N. (1997). Estrogenic activity in five United Kingdom rivers detected by measurement of vitellogenesis in caged male trout. *Environ. Toxicol. Chem.* **16**, 534-542.

Hawkins, M. B., Thornton, J. W., Crews, D., Skipper, J. K., Dotte, A., and Thomas, P. (2000). Identification of a third distinct estrogen receptor and reclassification of estrogen receptors in teleosts. *Proc. Natl. Acad. Sci. USA* **97**, 10751-10756.

Hayano, T. and Kikuchi, M. (1995). Molecular cloning of the cDNA encoding a novel protein disulfide isomerase-related protein (PDIR). *FEBS Lett.* **372**, 210-214.

Hemmer, M. J., Hemmer, B. L., Bowman, C. J., Kroll, K. J., Folmar, L. C., Marcovich, D., Hoglund, M. D., and Denslow, N. D. (2001). Effects of p-nonylphenol, methoxychlor, and endosulfan on vitellogenin induction and expression in sheepshead minnow (*Cyprinidon variegatus*). *Environ. Toxicol. Chem.* **20**, 336-343.

Heppell, S. A., Denslow, N. D., Folmar, L. C., and Sullivan, C. V. (1995). Universal assay of vitellogenin as a biomarker for environmental estrogens. *Environ. Health Perspect.* **103**, 9-15.

Herbst, A. L. and Bern, H. A. (1981). Developmental Effects of Diethystilbestrol (DES) in Pregnancy, Thieme-Stratton, Inc., New York.

Herman, R. L. and Kincaid, H. L. (1988). Pathological effects of orally administered estradiol to rainbow trout. *Aquaculture* **72**, 165-172.

Herrin, D. L. and Schmidt, G. W. (1988). Rapid, reversible staining of northern blots prior to hybridization. *Biotechniques* **6**, 196-200.

Hightower, L. E. and Renfro, J. L. (1988). Recent applications of fish cell culture to biomedical research. *J. Exp. Zool.* **248**, 290-302.

Hsu, S. T., Ma, C. I., Hsu, S. K. H., Wu, S. S., Hsu, N. H. M., Yeh, C. C., and Wu, S. B. (1985). Discovery and epidemiology of PCB poisoning in Taiwan: a four-year followup. *Environ. Health Perspect.* **59**, 5-10.

Hunt, G. L. Jr., Wingfield, J. C., Newman, A., and Farner, D. S. (1980). Sex ratio of western gulls on Santa Barbara Island, California. *Auk.* **97**, 473-479.

Ignar-Trowbridge, D. M., Nelson, K. G., Bidwell, M. C., Curtis, S. W., Washburn, T. F., McLachlan, J. A., and Korach, K. S. (1992). Coupling of dual signaling pathways: epidermal growth factor action involves the estrogen receptor. *Proc. Natl. Acad. Sci. USA* **89** , 4658-4662.

Iida, K. I., Miyaishi, O., Iwata, Y., Kozaki, K. I., Matsuyama, M., and Saga, S. (1996). Distinct distribution of protein disulfide isomerase family proteins in rat tissues. *J. Histochem. Cytochem.* **44**, 751-759.

Improta, B. T., Whorton, A. R., Codazzi, F., York, J. D., Meyer, T., and McDonnell, D. P. (1999). Estrogen-induced activation of mitogen-activated protein kinase requires mobilization of intracellular calcium. *Proc. Natl. Acad. Sci. USA* **96**, 4686-4691.

Iwata, H., Tanabe, S., Sakai, N., and Tatsukawa, R. (1993). Distribution of persistent organochlorines in the oceanic air and surface seawater and the role on their global transport and fate. *Environ. Sci. Technol.* **27**, 1080-1098.

Jansen, H. T., Cooke, P. S., Porcelli, J., Liu, T.-C., and Hansen, L. G. (1993). Estrogenic and antiestrogenic actions of PCBs in the female rat: In vitro and in vivo studies. *Reprod. Toxicol.* **7**, 237-248.

Jensen, E.V. and Jacobson, H.I. (1960). Fate of steroid estrogens in target tissues. In: Biological Activities of Steroids in Relation to Cancer (G. Pincus and E.P. Vollmer, Eds.), pp. 161-178. Academic Press, New York.

Jobling, S., Nolan, M., Tyler CR, Brighty, G. C., and Sumpter, J. P. (1998). Widespread sexual disruption in wild fish. *Environ. Sci. Technol.* **32**, 2498-2506.

Jobling, S., Reynolds, T., White, R., Parker, M. G., and Sumpter, J. P. (1995). A variety of environmentally persistent chemicals, including some phthalate plasticizers, are weakly estrogenic. *Environ. Health Perspect.* **103**, 582-587.

Jobling, S., Sheahan, D. A., Osborne, J. A., Matthiessen, P., and Sumpter, J. P. (1996). Inhibition of testicular growth in rainbow trout (*Oncorhynchus mykiss*) exposed to estrogenic alkylphenolic chemical. *Environ. Toxicol. Chem.* **15**, 194-202.

Jobling, S. and Sumpter, J. P. (1993). Detergent components in sewage effluent are weakly oestrogenic to fish: An in vitro study using rainbow trout (*Oncorhynchus mykiss*) hepatocytes. *Aquat. Toxicol.* **27**, 361-372.

Johnson, L. L., Casillas, E., Collier, T. K., McCain, B. B., and Varanasi, U. (1988). Contaminant effects on ovarian development in English sole (*Parophrys vetulus*) from Puget Sound, Washington. *Can. J. Fish Aquat. Sci.* **45**, 2133-2146.

Joseph, D. R. (1994). Structure, function, and regulation of androgen-binding protein/sex hormone-binding globulin. *Vit. Horm.* **49**, 197-280.

Kaplitt, M. G., Kleopoulos, S. P., Pfaff, D. W., and Mobbs, C. V. (1993). Estrogen increases HIP-70/PLC-a messenger ribonucleic acid in the rat uterus and hypothalamus. *Endocrinology* **133**, 99-104.

Kato, S., Endoh, H., Masuhiro, Y., Kitamoto, T., Uchiyama, S., Sasaki, H., Masushige, S., Gotoh, Y., Nishida, E., Kawashima, H., Metzger, D., and Chambon, P. (1995). Activation of the estrogen receptor through phosphorylation by mitogen-activated protein kinase. *Science* **270**, 1491-1494.

Kavlock, R. J., Daston, G. P., DeRosa, C., Fenner-Crisp, P., Gray, L. E. Jr., Kaattari, S., Lucier, G. W., Luster, M., Mac, M. J., Maczka, C., Miller, R., Moore, J., Rolland RM, Scott, G., Sheehan DM, Sinks, T., and Tilson, H. A. (1996). Research needs for the risk assessment of health and environmental effects of endocrine disruptors: A report of the U.S. EPA-sponsored workshop. *Environ. Health Perspect.* **104**, 715-740.

King, W. J. and Greene, G. L. (1984). Monoclonal antibodies localize oestrogen receptor in the nuclei of target cells. *Nature* **307**, 745-747.

Klaunig, J. E., Ruch, R. J., and Goldblatt, P. J. (1985). Trout hepatocyte culture: isolation and primary culture. *In Vitro Cell. Dev. Biol.* **21**, 221-228.

Kocal, T., Quinn, B. A., Smith, I. R., Ferguson, H. W., and Hayes, M. A. (1988). Use of trout serum to prepare primary attached monolayer cultures of hepatocytes from rainbow trout (*Salmo gairdneri*). *In Vitro Cell. Dev. Biol.* **24**, 304-308.

Koike, S., Sakai, M., and Muramatsu, M. (1987). Molecular cloning and characterization of rat estrogen receptor cDNA. *Nuc. Acids Res.* **15**, 2499-2513.

Korte, J. J., Kahl, M. D., Jensen, K. M., Pasha, M. S., Parks, L. G., LeBlanc, G. A., and Ankley, G. T. (2000). Fathead minnow vitellogenin: Complementary DNA sequence and messenger RNA and protein expression after 17 β-estradiol treatment. *Environ. Toxicol. Chem.* **19**, 972-981.

Krishnan, V., Porter, W., Santostefano, M., Wang, X., and Safe, S. (1995). Molecular mechanism of inhibition of estrogen-induced cathepsin D gene expression by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in MCF-7 cells. *Mol. Cell. Biol.* **15**, 6710-6719.

Kubeck, A. and Naylor, C. G. (1990). Trace analysis of alkylphenol ethoxylates. *J. Am. Oil Chem. Soc.* **67**, 400-405.

Kuiper, G. G. J. M., Enmark, E., Pelto-Huikko, M., Nilsson, S., and Gustafsson, J. A. (1996). Cloning of a novel estrogen receptor expressed in rat prostate and ovary. *Proc. Natl. Acad. Sci. USA* **93**, 5925-5930.

Kumar, V. and Chambon, P. (1988). The estrogen receptor binds tightly to its responsive element as a ligand-induced homodimer. *Cell* **55**, 145-156.

LaFleur, G.J.Jr., Byrne, B.M., Haux, C., Greenburg, R.M., and Wallace, R.A. (1995b). Liver-derived cDNAs: Vitellogenins and vitelline envelope protein precursor (choriogenins). In: International Symposium on Reproduction Physiology of Fish, July 7-12, 1995 (F.W. Goetz and P. Thomas, Eds.), pp. 336-338. Fish Symposium 95 Publishers, University of Texas, Austin, TX.

LaFleur, G. J. Jr., Byrne, B. M., Kanungo, J., Nelson, L. D., Greenburg, R. M., and Wallace, R. A. (1995a). *Fundulus heteroclitus* vitellogenin: the deduced primary structure of a piscine precursor to noncrystalline, liquid-phase yolk protein. *J. Mol. Evol.* **41**, 505-521.

Landel, C. C., Kushner, P. J., and Greene, G. L. (1994). The interaction of human estrogen receptor with DNA is modulated by receptor-associated proteins. *Mol. Endocrinol.* **8**, 1407-1419.

Larsson, D. G. J., Hyllner, S. J., and Haux, C. (1994). Induction of vitelline envelope proteins by estradiol-17β in 10 teleost species. *Gen. Comp. Endocrinol.* **96**, 445-450.

Lazier, C. B., Lonergan, K., and Mommsen, T. P. (1985). Hepatic estrogen receptors and plasma estrogen-binding activity in the Atlantic salmon. *Gen. Comp. Endocrinol.* **57**, 234-245.

Le Goff, P., Montano, M. M., Schodin, D. J., and Katzenellenbogen, B. S. (1994). Phosphorylation of the human estrogen receptor: Identification of hormone-regulated sites and examination of their influence on transcriptional activity. *J. Biol. Chem.* **269**, 4458-4466.

Le Guellec, K., Lawless, K., Valotaire, Y., Kress, M., and Tenniswood, M. (1988). Vitellogenin gene expression in male rainbow trout (*Salmo gairdneri*). *Gen. Comp. Endocrinol.* **71**, 359-371.

Lech, J. J., Lewis, S. K., and Ren, L. (1996). In vivo estrogenic activity of nonylphenol in rainbow trout. *Fund. Appl. Toxicol.* **30**, 229-232.

Lee, B. and Peart, T. E. (1995). Determination of 4-NP in effluent and sludge from sewage treatment plants. *Anal. Chem.* **67**, 1967-1980.

Lee, K. B. H., Lim, E. H., Lam, T. J., and Ding, J. L. (1992). Vitellogenin diversity in the perciformes. *J. Exp. Zool.* **264**, 100-106.

Lee, P. C., Patra, S. C., Stelloh, C. T., Lee, W., and Struve, M. (1996b). Interaction of nonylphenol and hepatic CYP1A in rats. *Biochem. Pharmacol.* **52**, 885-889.

Lee, P. C., Patra, S. C., and Struve, M. (1996a). Modulation of rat hepatic CYP3A by nonylphenol. *Xenobiotica* **26**, 831-838.

Li, H. C., Dehal, S. S., and Kupfer, D. (1995). Induction of the hepatic CYP2B and CYP3A enzymes by the proestrogenic pesticide methoxychlor and by DDT in the rat. Effects on methoxychlor metabolism. *J. Biochem. Toxicol.* **10**, 51-61.

Li, H. C. and Kupfer, D. (1998). Mechanism of induction of rat hepatic CYP2B and 3A by the pesticide methoxychlor. *J. Biochem. Mol. Toxicol.* **12**, 315-323.

Li, H. C., Mani, C., and Kupfer, D. (1993). Reversible and time-dependent inhibition of the hepatic cytochrome P450 steroidal hydroxylases by the proestrogenic pesticide methoxychlor in rat and human. *J. Biochem. Toxicol.* **8**, 195-206.

Liang, P. and Pardee, A. B. (1992). Differential display of eucaryotic messenger RNA by means of the polymerase chain reaction. *Science* **257**, 967-971.

Liang, P., Zhu, W., Zhang, X., Guo, Z., O'Connell, R. P., Averboukh, L., Wang, F., and Pardee, A. B. (1994). Differential display using one-base anchored oligo-dT primers. *Nuc. Acids Res.* **22**, 5763-5764.

Lim, E. H., Ding, J. L., and Lam, T. J. (1991). Estradiol-induced vitellogenin gene expression in a teleost fish, *Oreochromis aureus*. *Gen. Comp. Endocrinol.* **82**, 206-214.

Linnik, K. M. and Herscovitz, H. (1998). Multiple molecular chaperones interact with apolipoprotein B during its maturation. *J. Biol. Chem.* **273**, 21368-21373.

Lipsky, M. M., Sheridan, T. R., Bennett, R. O., and May, E. B. (1986). Comparison of trout hepatocyte culture on different substrates. *In Vitro Cell. Dev. Biol.* **22**, 360-362.

Loomis, A. K. and Thomas, P. (2000). Effects of estrogens and xenoestrogens on androgen production by Atlantic croaker testes in vitro: evidence for a nongenomic action mediated by an estrogen membrane receptor. *Biol. Reprod.* **62**, 995-1004.

Lucas, P. C. and Granner, D. K. (1992). Hormone response domains in gene transcription. *Annu. Rev. Biochem.* **61**, 1131-1173.

MacLatchy, D. L. and Van Der Kraak, G. J. (1995). The phytoestrogen β-sitosterol alters the reproductive endocrine status of goldfish. *Toxicol. Appl. Pharmacol.* **134**, 305-312.

Mader, S., Chambon, P., and White, J. H. (1993). Defining a minimal estrogen receptor DNA binding domain. *Nuc. Acids Res.* **21**, 1125-1132.

Madsen, S. S., Mathiesen, A. B., and Korsgaard, B. (1997). Effects of 17β-estradiol and 4-nonylphenol on smoltification and vitellogenesis in Atlantic salmon (*Salmo salar*). *Fish Physiol. Biochem.* **17**, 303-312.

Martin, L. and Claringbold, P. J. (1958). A highly sensitive assay for oestrogens. *Nature* **181**, 620-621.

Matsubara, T., Ohkubo, N., Andoh, T., Sullivan, C. V., and Hara, A. (1999). Two forms of vitellogenin, yielding two distinct lipovitellins, play different roles during oocyte maturation and early development of Barfin flounder, *Verasper moseri*, a marine teleost that spawns pelagic eggs. *Dev. Biol.* **213**, 18-32.

Mazzarella, R. A., Srinivasan, M., Haugejorden, S. M., and Green, M. (1990). ERp72, an abundant luminal endoplasmic reticulum protein, contains three copies of the active site sequences of protein disulfide isomerase. *J. Biol. Chem.* **265**, 1094-1101.

McLachlan, J. A., Newbold, R. R., and Bullock, B. C. (1975). Reproductive tract lesions in male mice exposed prenatally to diethylstilbestrol. *Science* **190**, 991-992.

Mellanen, P., Petanen, T., Lehtimaki, J., Makela, S., Bylund, G., Holmbom, B., Mannila, E., Oikari, A., and Santti, R. (1996). Wood-derived estrogens: Studies in vitro with breast cancer cell lines and in vivo in trout. *Toxicol. Appl. Pharmacol.* **136**, 381-388.

Mikawa, N., Hirono, I., and Aoki, T. (1996). Structure of medaka transferrin gene and its 5'-flanking region. *Mol. Mar. Biol. Biotechnol.* **5**, 225-229.

Miksicek, R. J. (1995). Estrogenic flavonoids: Structural requirements for biological activity. *Proc. Soc. Exp. Biol. Med.* **208**, 44-50.

Miles, C. J. and Pfeuffer, R. J. (1997). Pesticides in canals of south Florida. *Arch. Environ. Contam. Toxicol.* **32**, 337-345.

Mills, L. J., Gutjahr-Gobell, R. E., Haebler, R. A., Horowitz, D. J. B., Jayaraman, S., Pruell, R. J., McKinney, R. A., Gardner, G. R., and Zaroogian, G. E. (2001). Effects of estrogenic (o,p'-DDT; octylphenol) and anti-androgenic (p,p'-DDE) chemicals on indicators of endocrine status in juvenile male summer flounder (*Paralichthys dentatus*). *Aquat. Toxicol.* **52**, 157-176.

Milligan, S. R., Balasubramanian, A. V., and Kalita, J. C. (1998). Relative potency of xenobiotic estrogens in an acute in vivo mammalian assay. *Environ. Health Perspect.* **106**, 23-26.

Mommsen, T.P., Moon, T.W., and Walsh, P.J. (1994). Hepatocytes: Isolation, maintenance and utilization. In: Analytical Techniques (P.W. Hochachka and T.P. Mommsen, Eds.), pp. 355-373. Elsevier, Amsterdam.

Mommsen, T.P. and Walsh, P.J. (1988). Vitellogenesis and oocyte assembly. In: Fish Physiology (W.S. Hoar and D.J. Randall, Eds.), pp. 347-406. Academic Press, San Diego.

Moon, T. W., Walsh, P. J., and Mommsen, T. P. (1985). Fish hepatocytes: A model metabolic system. *Can. J. Fish Aquat. Sci.* **42**, 1772-1782.

Morley, P., Whitfield, J. F., Vanderhyden, B. C., Tsang, B. K., and Schwartz, J. L. (1992). A new, nongenomic estrogen action: the rapid release of intracellular calcium. *Endocrinology* **131**, 1305-1312.

Mouchel, N., Trichet, V., Betz, A., Le Pennec, J. P., and Wolff, J. (1996). Characterization of vitellogenin from rainbow trout (*Oncorhynchus mykiss*). *Gene* **174**, 59-64.

Munkittrick, K. R., Van Der Kraak, G. J., McMaster, M. E., Portt, C. B., Van Den Heuvel, M. R., and Servos, M. (1994). Survey of receiving-water environmental impacts associated with discharges from pulp mills. 2. Gonad size, liver size,

hepatic EROD activity and plasma sex steroid levels in white sucker. *Environ. Toxicol. Chem.* **13**, 1089-1101.

Mylchreest, E., Cattley, R. C., and Foster, P. M. (1998). Male reproductive tract malformations in rats following gestational and lactational exposure to di (n-butyl) phthalate: An antiandrogenic mechanism? *Toxicol. Sci.* **43**, 47-60.

Mylchreest, E., Sar, M., Cattley, R. C., and Foster, P. M. (1999). Disruption of androgen-regulated male reproductive development by di (n-butyl) phthalate during late gestation in rats is different from flutamide. *Toxicol. Appl. Pharmacol.* **156**, 81-95.

Nagel, S. C., vom Saal, F. S., Thayer, K. A., Dhar, M. D., Boechler, M., and Welshons, W. V. (1997). Relative binding affinity-serum modified access (RBA-SMA) assay predicts the relative in vivo bioactivity of the xenoestrogens bisphenol A and octylphenol. *Environ. Health Perspect.* **105**, 70-76.

Nagel, S. C., vom Saal, F. S., and Welshons, W. V. (1998). The effective free fraction estradiol and xenoestrogens in human serum measured by whole cell uptake assays: Physiology of delivery modifies estrogenic activity. *Proc. Soc. Exp. Biol. Med.* **217**, 300-309.

Naylor, C. G., Mieure, J. P., Adams, W. J., Weeks, J. A., Castaldi, F. J., Ogle, L. D., and Romano, R. R. (1992). Alkylphenol ethoxylates in the environment. *J. Am. Oil Chem. Soc.* **69**, 695-703.

Newbold, R. R. (1995). Cellular and molecular effects of developmental exposure to diethylstilbestrol: implications for other environmental estrogens. *Environ. Health Perspect.* **103**, 83-87.

Nichols, K. M., Snyder, E. M., Snyder, S. A., Pierens, S. L., Miles-Richardson, S. R., and Giesy, J. P. (2001). Effects of nonylphenol ethoxylate exposure on reproductive output and bioindicators of environmental estrogen exposure in fathead minnows, *Pimephales promelas*. *Environ. Toxicol. Chem.* **20**, 510-522.

Nimrod, A. C. and Benson, W. H. (1996a). Environmental estrogenic effects of alkylphenol ethoxylates. *Crit. Rev. Toxicol.* **26**, 335-364.

Nimrod, A. C. and Benson, W. H. (1996b). Estrogenic responses to xenobiotics in Channel catfish (*Ictalurus punctatus*). *Mar. Environ. Res.* **42**, 155-160.

Nimrod, A. C. and Benson, W. H. (1997). Xenobiotic interaction with and alteration of Channel catfish estrogen receptor. *Toxicol. Appl. Pharmacol.* **147**, 381-390.

Noiva, R., Freedman, R. B., and Lennarz, W. J. (1993). Peptide binding to protein disulfide isomerase occurs at a site distinct from the active sites. *J. Biol. Chem.* **268**, 19210-19217.

Olea, N., Pulgar, R., Perez, P., Olea-Serrano, F., Rivas, A., Novillo-Fertrell, A., Pedraza, V., Soto, A. M., and Sonnenschein, C. (1996). Estrogenicity of resin-based composites and sealants used in dentistry. *Environ. Health Perspect.* **104**, 298-315.

Oppen-Berntsen, D. O., Olsen, S. O., Rong, C. J., Taranger, G. L., Swanson, P., and Walther, B. T. (1994). Plasma levels of eggshell zr-proteins, estradiol-17β, and gonadotropins during an annual reproductive cycle of Atlantic salmon (*Salmo salar*). *J. Exp. Zool.* **268**, 59-70.

Ostrander, G. K., Blair, J. B., Stark, B. A., Marley, G. M., Bales, W. D., Veltri, R. W., Hinton, D. E., Okihiro, M., Ortego, L. S., and Hawkins, W. E. (1995). Long-term primary culture of epithelial cells from rainbow trout (*Oncorhynchus mykiss*) liver. *In Vitro Cell. Dev. Biol.* **31**, 367-378.

Pace, P., Taylor, J. A., Suntharalingam, S., Coombes, R. C., and Ali, S. (1997). Human estrogen receptor β binds DNA in a manner similar to and dimerizes with estrogen receptor α. *J. Biol. Chem.* **272**, 25832-25838.

Paech, K., Webb, P., Kuiper, G. G. J. M., Nilsson, S., Gustafsson, J. A., Kushner, P. J., and Scanlan, T. S. (1997). Differential ligand activation of estrogen receptors ER α and ER β at AP1 sites. *Science* **277**, 1508-1510.

Pakdel, F., Feon, S., Le Gac, F., Le Menn, F., and Valotaire, Y. (1991). In vivo estrogen induction of hepatic estrogen receptor mRNA and correlation with vitellogenin mRNA in rainbow trout. *Mol. Cell. Endocrinol.* **75**, 205-212.

Pakdel, F., Le Gac, F., Le Goff, P., and Valotaire, Y. (1990). Full-length sequence and in vitro expression of rainbow trout estrogen receptor cDNA. *Mol. Cell. Endocrinol.* **71**, 195-204.

Pakdel, F., Le Guellec, C., Vaillant, C., Le Roux, M. G., and Valotaire, Y. (1989). Identification and estrogen induction of two estrogen receptors (ER) messenger ribonucleic acids in the rainbow trout liver: sequence homology with other ERs. *Mol. Endocrinol.* **3**, 44-51.

Palmer, B.D. and Selcer, K.W. (1996). Vitellogenin as a biomarker for xenobiotic estrogens: a review. In: Environmental Toxicology and Risk Assessment: Biomarkers and Risk Assessment (D.A. Bengston and D.S. Henshel, Eds.), pp. 3-22. American Society for Testing and Materials, Philadelphia, PA.

Panter, G. H., Thompson, R. S., and Sumpter, J. P. (1998). Adverse reproductive effects in male fathead minnows (*Pimephales promelas*) exposed to environmentally relevant concentrations of the natural oestrogens, oestradiol and oestrone. *Aquat. Toxicol.* **42**, 243-253.

Patino, R., Xia, Z., Gale, W. L., Wu, C., Maule, A. G., and Chang, X. (2000). Novel transcripts of the estrogen receptor α gene in channel catfish. *Gen. Comp. Endocrinol.* **120**, 314-325.

Pelissero, C., Flouriot, G., Foucher, J. L., Bennetau, B., Dunogues, J., Le Gac, F., and Sumpter, J. P. (1993). Vitellogenin synthesis in cultured hepatocytes; an in vitro test for the estrogenic potency of chemicals. *J. Steroid Biochem. Mol. Biol.* **44**, 263-272.

Peterson, D. N., Tkalcevic, G. T., Koza-Taylor, P. H., Turi, T. G., and Brown, T. A. (1998). Identification of estrogen receptor β_2, a functional variant of estrogen receptor β expressed in normal rat tissues. *Endocrinology* **139**, 1082-1092.

Petit, F., Le Goff, P., Cravedi, J. P., Valotaire, Y., and Pakdel, F. (1997). Two complementary bioassays for screening the estrogenic potency of xenobiotics: recombinant yeast for trout estrogen receptor and trout hepatocyte cultures. *J. Mol. Endocrinol.* **19**, 321-335.

Pilat, M. J., Hafner, M. S., Kral, L. G., and Brooks, S. C. (1993). Differential induction of pS2 and cathepsin D mRNAs by structurally altered estrogens. *Biochemistry* **32**, 7009-7015.

Purdom, C. E., Hardiman, P. A., Bye, V. J., Eno, N. C., Tyler, C. R., and Sumpter, J. P. (1994). Estrogenic effects of effluents from sewage treatment works. *Chem. Ecol.* **8**, 275-285.

Rapaport, R. A., Urban, N. R., Capel, P. D., Baker, J. E., Looney, B. B., Eisenreich, S. J., and Gorham, E. (1985). "New" DDT inputs to North America: Atmospheric deposition. *Chemosphere* **14**, 1167-1173.

Ratnasabapathy, R., Tom, M., and Post, C. (1997). Modulation of the hepatic expression of the estrogen-regulated mRNA stabilizing factor by estrogenic and antiestrogenic nonsteroidal xenobiotics. *Biochem. Pharmacol.* **53**, 1425-1434.

Rice, K. G., Percival, H. F., Woodward, A. R., Abercrombie, C. L., Wilkinson, P. M. (1996). Clutch viability, population trends, and nesting female demographics. In: Effects of Environmental Contaminants on the Demographics and Reproduction of Lake Apopka's Alligators and Other Taxa. (K. G. Rice and H. F. Percival, Eds.) **53**, pp. 1-83. Florida Cooperative Fish and Wildlife Research Unit, U.S. Biological Service.

Rogan, W. J., Gladen, B. C., Hung, K., Koong, S., Shih, L., Taylor, J. S., Wu, Y., Yang, D., Ragan, N. B., and Hsu, C. (1988). Congenital poisoning by polychlorinated biphenyls and their contaminants in Taiwan. *Science* **241**, 334-336.

Rogan, W. J., Gladen, B. C., McKinney, J. D., Carreras, N., Hardy, P., Thullen, J., Tingelstad, J., and Tully, M. (1987). Polychlorinated biphenyls (PCBs) and dichlorodiphenyl dichloroethene (DDE) in human milk: Effects on growth, morbidity, and duration of lactation. *Am. J. Public Health* **77**, 1294-1297.

Rubin, B. L., Dorfman, A. S., Black, L., and Dorfman, R. I. (1951). Bioassay of estrogens using the mouse uterine response. *Endocrinology* **49**, 429-439.

Ryffel, G. U. (1978). Synthesis of vitellogenin, an attractive model for investigating hormone-induced gene activation. *Mol. Cell. Endocrinol.* **12**, 237-246.

Saceda, M., Lindsey, R. K., Solomon, H., Angeloni, S. V., and Martin, M. B. (1998). Estradiol regulates estrogen receptor mRNA stability. *J. Steroid Biochem. Mol. Biol.* **66**, 113-120.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, New York.

Schane, H. P., Anzalone, A. J., and Potts, G. O. (1972). A model for the evaluation of estrogens: Withdrawal bleeding in ovariectomized rhesus monkeys. *Fertil. Steril.* **23**, 745-750.

Schlenk, D., Stressor, D. M., Rimoldi, J., Arcand, L., McCants, J. C., Nimrod, A. C., and Benson, W. H. (1998). Biotransformation and estrogenic activity of methoxychlor and its metabolites in channel catfish (*Ictalurus punctatus*). *Mar. Environ. Res.* **46**, 159-162.

Schmid, T., Gonzalez-Valero, J., and Dietrich, D. R. (2001). Determination of the half-life of vitellogenin as an indicator for endocrine modulation. *The Toxicologist* **60** (Suppl.), 164-165.

Segner, H. (1998). Isolation and primary culture of teleost hepatocytes. *Comp. Biochem. Physiol.* **120** , 71-81.

Segner, H., Blair, J. B., Wirtz, G., and Miller, M. R. (1994). Cultured trout liver cells: utilitization of substrates and response to hormones. *In Vitro Cell. Dev. Biol.* **30A**, 306-311.

Sekela, M., Brewer, R., Moyle, G., and Tuominen, T. (1999). Occurrence of an environmental estrogen (4-nonylphenol) in sewage treatment plant effluent and the aquatic receiving environment. *Water Sci. Tech.* **39**, 217-220.

Servos, M. R. (1999). Review of the aquatic toxicity, estrogenic responses and bioaccumulation of alkylphenols and alkylphenol polyethoxylates. *Water Qual. Res. J. Can.* **34**, 123-177.

Shapiro, D. J., Barton, M. C., McKearin, D. M., Chang, T. C., Lew, D., Blume, J., Nielsen, D. A., and Gould, L. (1989). Estrogen regulation of gene transcription and mRNA stability. *Rec. Prog. Horm. Res.* **45**, 29-58.

Sharpe, R. M., Fisher, J. S., Millar, M. M., Jobling, S., and Sumpter, J. P. (1995). Gestational and lactational exposure of rats to xenoestrogens results in reduced testicular size and sperm production. *Environ. Health Perspect.* **103**, 1136-1143.

Shelby, M. D., Newbold, R. R., Tully, D. B., Chae, K., and Davis, V. L. (1996). Assessing environmental chemicals for estrogenicity using a combination of in vitro and in vivo assays. *Environ. Health Perspect.* **104**, 1296-1300.

Shibata, H., Spencer, T. E., Onate, S. A., Jenster, G., Tsai, S. Y., Tsai, M. J., and O'Malley, B. W. (1997). Role of co-activators and co-repressors in the mechanism of steroid/thyroid receptor action. *Rec. Prog. Horm. Res.* **52**, 141-165.

Smith, J. S. and Thomas, P. (1991). Changes in hepatic estrogen receptor concentrations during the annual reproductive and ovarian cycles of a marine teleost, the spotted sea trout, *Cynoscion nebulosus*. *Gen. Comp. Endocrinol.* **81**, 234-245.

Snyder SA, Keith, T. L., Verbrugge DA, Snyder, E. M., Gross, T. S., Kannan, K., and Giesy, J. P. (1999). Analytical methods for detection of selected estrogenic compounds in aqueous mixtures. *Environ. Sci. Technol.* **33**, 2814-2820.

Socorro, S., Power, D. M., Olsson, P. E., and Canario, A. V. (2000). Two estrogen receptors expressed in the teleost fish, *Sparus aurata*: cDNA cloning, characterization, and tissue distribution. *J. Endocrinol.* **166**, 293-306.

Soto, A. M., Chung, K. L., and Sonnenschein, C. (1994). The pesticides endosulfan, toxaphene, and dieldrin have estrogenic effects on human estrogen-sensitive cells. *Environ. Health Perspect.* **102**, 380-383.

Soto, A. M., Sonnenschein, C., Chung, K. L., Fernandez, M. F., Olea, N., and Olea-Serrano, F. (1995). The E-SCREEN assay as a tool to identify estrogens: an update on estrogenic environmental pollutants. *Environ. Health Perspect.* **103**, 113-122.

Soto, A. M., Sonnenschein, C., Murray, M. K., and Michaelson, C. L. (1997). Developing a marker of exposure to xenoestrogen mixtures in human serum. *Environ. Health Perspect.* **105** (Suppl. 3), 647-654.

Spee, P., Subjeck, J., and Neefjes, J. (1999). Identification of novel peptide binding proteins in the endoplasmic reticulum: ERp72, calnexin, and grp170. *Biochemistry* **38**, 10559-10566.

Srivastava, R. A. K., Srivastava, N., Averna, M., Lin, R. C., Korach, K. S., Lubahn, D. B., and Schonfeld G (1997). Estrogen up-regulates apolipoprotein E (ApoE) gene expression by increasing ApoE mRNA in the translating pool via the estrogen receptor α-mediated pathway. *J. Biol. Chem.* **272**, 33360-33366.

Sumpter, J. P. and Jobling, S. (1995). Vitellogenesis as a biomarker for estrogenic contamination of the aquatic environment. *Environ. Health Perspect.* **103**, 173-178.

Tabak, H. H., Bloomhuff, R. N., and Bunch, R. L. (1981). Steroid hormones as water pollutants. II. Studies on the persistence and stability of natural urinary and synthetic ovulation-inhibiting hormones in untreated and treated wastewaters. *Dev. Ind. Microbiol.* **22**, 497-519.

Talmadge, S. S. (1994). Environmental and human safety of major surfactants: alcohol ethoxylates and alkylphenol ethoxylates. Lewis Publishers, Boca Raton, FL.

Tan, N. S., Lam, T. J., and Ding, J. L. (1996). Transcription regulatory signals in the 5' and 3' regions of the *Oreochromis aureus* estrogen receptor gene. *Mol. Cell. Endocrinol.* **123**, 149-161.

Tata, J. R. (1976). The expression of the vitellogenin gene. *Cell* **9**, 1-14.

Tata, J. R. and Smith, D. F. (1979). Vitellogenesis: a versatile model for hormonal regulation of gene expression. *Rec. Prog. Horm. Res.* **35**, 47-95.

Tchoudakova, A., Pathak, S., and Callard, G. V. (1999). Molecular cloning of an estrogen receptor β subtype from the goldfish, *Carassius auratus*. *Gen. Comp. Endocrinol.* **113**, 388-400.

Teng, C. (1995). Mouse lactoferrin gene: a marker for estrogen and epidermal growth factor. *Environ. Health Perspect.* **103** (Suppl 7), 17-20.

Teng, C. T. (1999). Regulation of lactoferrin gene expression by estrogen and epidermal growth factor: molecular mechanism. *Cell Biochem. Biophys.* **31**, 49-64.

Thorpe, K. L., Hutchinson, T. H., Hetheridge, M. J., Sumpter, J. P., and Tyler, C. R. (2000). Development of an in vivo screening assay for estrogenic chemicals using juvenile rainbow trout (*Oncorhynchus mykiss*). *Environ. Toxicol. Chem.* **19**, 2812-2820.

Thummel, C. S. (1995). From embryogenesis to metamorphosis: the regulation and function of drosophilia nuclear receptor superfamily members. *Cell* **83**, 871-877.

Toppari, J., Larsen, J. C., Christiansen, P., Giwercman, A., Grandjean, P., Guillette, L. J. Jr., Jegou, B., Jensen, T. K., Jouannet, P., Keiding, N., Leffers, H., McLachlan, J. A., Meyer, O., Muller, J., Rajpert-De, M. E., Scheike, T., Sharpe, R., Sumpter, J., and Skakkebaek, N. E. (1996). Male reproductive health and environmental xenoestrogens. *Environ. Health Perspect.* **104** (Suppl 4), 741-803.

Truss, M. and Beato, M. (1993). Steroid hormone receptors: interaction with deoxyribonucleic acid and transcription factors. *Endocrin. Rev.* **14**, 459-479.

Tsai, M. J. and O'Malley, B. W. (1994). Molecular mechanisms of action of steroid/thyroid receptor superfamily members. *Annu. Rev. Biochem.* **63**, 451-486.

Tsibris, J. C. M., Hunt, L. T., Ballejo, G., Barker, W. C., Toney, L. J., and Spellacy, W. N. (1989). Selective inhibition of protein disulfide isomerase by estrogens. *J. Biol. Chem.* **264**, 13967-13970.

U.S.Environmental Protection Agency. (1977). Chronic toxicity of methoxychlor, malathion, and carbofuran to sheepshead minnow (*Cyprinidon variegatus*). EPA-600/3-77-059. Gulf Breeze, FL, United States Environmental Protection Agency.

Van, P. N., Rupp, K., Lampen, A., and Soling, H. D. (1993). CaBP2 is a rat homolog of ERp72 with protein disulfide isomerase activity. *Eur. J. Biochem.* **213**, 789-795.

Van Der Kraak, G. J., Munkittrick, K. R., McMaster, M. E., Portt, C. B., and Chang, J. P. (1992). Exposure to bleached kraft pulp mill effluent disrupts the pituitary-gonadal axis of white sucker at multiple sites. *Toxicol. Appl. Pharmacol.* **115**, 224-233.

van het Schip, F. D., Samallo, J., Broos, J., Ophuis, J., Mojet, M., Gruber, M., and Ab, G. (1987). Nucleotide sequence of a chicken vitellogenin gene and derived amino acid sequence of the encoded yolk precursor protein. *J. Mol. Biol.* **190**, 245-260.

Verdeal, K., Brown, R. R., Richardson, T., and Ryan, D. S. (1980). Affinity of phytoestrogens for estradiol-binding proteins and effect of coumestrol on growth of 7,12-demethylbenz[a]anthracene-induced rat mammary tumors. *J. Natl. Cancer Inst.* **64**, 285-290.

vom Saal, F. S., Nagel, S. C., Palanza, P., Boechler, M., Parmigiani, S., and Welshons, W. V. (1995). Estrogenic pesticides: Binding relative to estradiol in MCF-7 cells and effects of exposure during fetal life on subsequent territorial behaviour in male mice. *Toxicol. Lett.* **77**, 343-350.

vom Saal, F. S., Timms, B. G., Montano, M. M., Palanza, P., Thayer, K. A., Nagel, S. C., Dhar, M. D., Ganjam, V. K., Parmigiani, S., and Welshons, W. V. (1997). Prostate enlargement in mice due to fetal exposure to low doses of estradiol and diethylstilbestrol and opposite effects at high doses. *Proc. Natl. Acad. Sci. USA* **94**, 2056-2061.

Vonier, P. M., Crain, D. A., McLachlan, J. A., Guillette, L. J. Jr., and Arnold, S. F. (1996). Interaction of environmental chemicals with the estrogen and progesterone receptors from the oviduct of the American alligator. *Environ. Health Perspect.* **104**, 1318-1322.

Wahli, W., Dawid, I. B., Ryffel, G. U., and Weber, R. (1981). Vitellogenesis and the vitellogenin gene family. *Science* **212**, 298-304.

Wallace, R. A. and Jared, D. W. (1968). Studies on amphibian yolk. VII. Serum phosphoprotein synthesis by vitellogenic females and estradiol-treated males of Xenopus laevis. *Can. J. Biochem.* **46**, 953-959.

Wang, S. Y., Smith, D. E., and Williams, D. L. (1983). Purification of avian vitellogenin III: comparison with vitellogenins I and II. *Biochemistry* **22**, 6202-6212.

Watson, C. S., Pappas, T. C., and Gametchu, B. (1995). The other estrogen receptor in the plasma membrane: implications for the actions of environmental estrogens. *Environ. Health Perspect.* **103**, 41-50.

Weisz, A. and Bresciani, F. (1993). Estrogen regulation of proto-oncogenes coding for nuclear proteins. *Crit. Rev. Oncog.* **4**, 361-388.

White, R., Jobling, S., Hoare, S. A., Sumpter, J. P., and Parker, M. G. (1994). Environmentally persistent alkylphenolic compounds are estrogenic. *Endocrinology* **135**, 175-182.

White, R., Lees, J. A., Needham, M., Ham, J., and Parker, M. (1987). Structural organization and expression of the mouse estrogen receptor. *Mol. Endocrinol.* **1**, 735-744.

Woodward, A. R. and Moore, C. T. (1990). Statewide Alligator Surveys. Final Report, pp. 1-24. Tallahassee, FL, Florida Game and Freshwater Fish Commission.

Xia, Z., Patino, R., Gale, W. L., Maule, A. G., and Densmore, L. D. (1999). Cloning, in vitro expression, and novel phylogenetic classification of a channel catfish estrogen receptor. *Gen. Comp. Endocrinol.* **113**, 360-368.

Xu, Q. Y. and Shively, J. E. (1988). Microsequence analysis of peptides and proteins. VII. Improved electroblotting of proteins onto membranes and derivatized glass-fiber sheets. *Anal. Biochem.* **170**, 19-30.

Yadetie, F., Arukwe, A., Goksoyr, A., and Male, R. (1999). Induction of hepatic estrogen receptor in juvenile Atlantic salmon in vivo by the environmental estrogen, 4-nonylphenol. *Sci. Total Environ.* **233**, 201-210.

Yamashita, F. and Hayashi, M. (1985). Fetal PCB syndrome: Clinical features, intrauterine growth retardation and possible alteration in calcium metabolism. *Environ. Health Perspect.* **59**, 41-45.

Young, L. J., Godwin, J., Grammer, M., Gahr, M., and Crews, D. (1995). Reptilian sex steroid receptors: amplification, sequence, and expression analysis. *J. Steroid Biochem. Mol. Biol.* **55**, 261-269.

Zabel, E. W., Walker, M. K., Hornung, M. W., Clayton, M. K., and Peterson, R. E. (1995). Interactions of polychlorinated dibenzo-p-dioxin, dibenzofuran, and biphenyl congeners for producing rainbow trout early life stage mortality. *Toxicol. Appl. Pharmacol.* **134** , 204-213.

Zacharewski, T. (1997). In vitro bioassays for assessing estrogenic substances. *Environ. Sci. Technol.* **31**, 613-623.

Zacharewski, T. (1998). Identification and assessment of endocrine disruptors: limitations of in vivo and in vitro assays. *Environ. Health Perspect.* **106**, 577-582.

BIOGRAPHICAL SKETCH

The author was born in Laramie, Wyoming, to two wonderful parents, Jim and Ellona Bowman, who raised their two sons to be successful in whatever life they choose. Through second grade he attended the University of Wyoming Preparatory School. At seven years of age his family moved to Tallahasssee, Florida where he attended Gilchrist Elementary School, Augusta Raa Middle School, and Lincoln High School. Immediately after high school the author moved to Gainesville, Florida. After four years of undergraduate work, he earned his Bachelor of Science degree in Interdisciplinary Science with highest honors from the University of Florida in 1996.

During his time at the University of Florida the author worked in various unrelated jobs in addition to laboratory work with Sheldon Schuster, Ph.D. in the Department of Biochemistry and Molecular Biology. For his undergraduate thesis, the author worked in the laboratory of Nihal Tumer, Ph.D. in the Department of Pharmacology and Therapeutics. Immediately after graduation, he started work on his Doctoral degree in August of 1996 in the College of Medicine Interdisciplinary Graduate Program. A couple of years later he was admitted to candidacy in the Department of Pharmacology and Therapeutics working with Nancy Denslow, Ph.D. After graduation, he plans to conduct post doctoral research at the CIIT Centers for Health Research in Research Triangle Park, North Carolina with Paul Foster, Ph.D.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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